METABOLISM OF ACETATE BY NITROBACTER AGILIS

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ABSTRACT

METABOLISM OF ACETATE BY *NITROBACTER AGILIS*

by Aline L. Garretson

Nitrobacter agilis was incapable of proliferation in a medium containing acetate (5 mM) as sole source of carbon when various inorganic and organic sources of nitrogen were substituted for nitrite. Furthermore, culture viability was not maintained over a 21 day growth period in an acetate medium supplemented with either glutamate or nitrate. Significant increases in cell number were not achieved with either ammonium or casein hydrolysate additions to a medium containing acetate. Repeated attempts to grow pure cultures of *N. agilis* heterotrophically in a medium containing acetate and casein hydrolysate were unsuccessful. Replication in a medium containing both acetate and nitrite approximated that observed with autotrophic cultures. The dry weight of cells grown in an autotrophic medium contained 0.3 to 0.4% poly-β-hydroxybutyrate (PHB) whereas cells grown in an autotrophic medium supplemented with acetate contained as much as 6 to 12% PHB. The increased synthesis of PHB was accompanied by both a decreased production of protein and reduced absorbance to dry weight ratios. The distribution pattern of ^{14}C in fractions of cells incorporating acetate-1-^{14}C remained unchanged when unlabeled bicarbonate was added but the total amount of ^{14}C assimilated was reduced 26% in all fractions. In contrast, the addition of

unlabeled acetate to a medium containing bicarbonate-^{14}C resulted in an 80% reduction in isotope assimilated while the distribution patterns remained the same. In cultures labeled with acetate-1-^{14}C approximately 30% of the radioactivity was located in PHB whereas 40% was found in the protein fraction. Intermediates of the tricarboxylic acid (TCA) cycle and certain amino acids and phosphorylated compounds located in the ethanol-soluble fraction of a cell suspension oxidizing nitrite were shown to be labeled within 5 sec after addition of acetate-2-^{14}C. The greatest amount of isotope recovered initially was in the carboxylic acids of the TCA cycle and in glutamate. Citrate and isocitrate were predominantly labeled while the radioactivity associated with malate remained at relatively low levels until nitrite was depleted. The sequential labeling patterns obtained during nitrite oxidation provided evidence for the operation of a TCA cycle, but ruled out the operation of either a glyoxylate bypass or the oxidative dicarboxylic acid cycle. However, when nitrite was exhausted. there were percentage increases in glyoxylate, malate, and succinate and decreases in citrate and isocitrate. It appears that the TCA cycle no longer is the dominant pathway for acetate metabolism once nitrite oxidation ceases and that glyoxylate becomes the principle intermediate labeled.

METABOLISM OF ACETATE BY *NITROBACTER AGILIS*

By

Aline L. Garretson

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To members of my family, for their constant encouragement, assistance, and understanding during the course of my doctoral program.

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INTRODUCTION

The accumulated data on the growth of obligate chemoautotrophs on organic substrates has not permitted an understanding of the failure for growth under heterotrophic conditions. The rapidity of nitrification in organically rich soils, dung heaps, sewage, and impure cultures has been cited by Lees (1954). Consequently, it follows that heterotrophic growth may be possible under limited and well-defined growth conditions in which a specific combination of organic substrates may prove essential.

Up to the present time, the supplementation of inorganic media with a single organic compound has not resulted in heterotrophic growth for a number of obligate chemolithotrophs, including Nitrobacter. On the other hand, complex supplements, such as yeast extract, have been shown to stimulate growth in an otherwise autotrophic medium. There has appeared only one report of Nitrobacter agilis growing heterotrophically (Smith and Hoare, 1968). This organism reportedly grew, albeit slowly, in the combined presence of acetate and casein hydrolysate and in the absence of any other form of nitrogen or carbon. However, heterotrophic growth of Nitrobacter in a similar medium has not been reported by any other investigators. Furthermore, short-

term studies with cells not adapted to heterotrophic growth on acetate have shown that acetate as sole source of carbon and energy does not support the replication of N. agilis (Delwiche and Finstein, 1965; Ida and Alexander, 1965). In the previous studies, neither the contribution of acetate as a precursor of cell metabolites, nor the metabolic function of casein hydrolysate has been ascertained.

The aim of this study was to elucidate the pathway(s) of carbon assimilation during growth in a medium containing acetate and to determine if acetate can serve as a source of energy for both carbon assimilation and cell growth. This dissertation reports the inability of N. agilis to grow in media containing acetate when any one of a variety of organic or inorganic nitrogenous compounds, including casein hydrolysate, was substituted for nitrite. When nitrite was present in the medium, growth approximated that of the control autotrophic cultures. From isotopic distribution and kinetic studies, using ^{14}C-labeled acetate, I concluded that acetate was not metabolized by either the glyoxylate bypass or the oxidative dicarboxylic acid cycle.

LITERATURE REVIEW

Structure and Biological Significance of Nitrobacter

N. agilis is a gram negative, flagellated bacterium originating in the soil. Electron microscopy demonstrated a characteristic peg-shape which is related to the polar arrangement of the plasma membrane intrusions (Murray and Watson, 1965). These intrusions consist of at least nine layers of plasma membrane, closely and regularly apposed which are arranged in two or three thicknesses over the poles of the cell. The plasma membrane uniquely possesses an extremely dense layer applied to the inside of the usual unit membrane. It is quite probable that this lammellar membrane system is associated with the cytochrome electron transport system involved in nitrite oxidation. Aleem and Nason (1959) have established the presence of this system in the particulate fraction of cell-free extracts.

The conversion of ammonia to nitrate is effected in nature by two groups of obligate aerobic, chemolithotrophic bacteria. Ammonia oxidation is accomplished by *Nitrosomonas* while nitrite is oxidized to nitrate by *Nitrobacter*. Only two species of *Nitrobacter* are recognized, *N. agilis* and *N.*

winogradskyi. The combined activities of the nitrifiers results in the oxidation of the ammonia released during the mineralization of organic matter. Consequently, in soils where nitrification is active, nitrate is the principal form of nitrogen that is available for the growth of plants. The nitrifiers which are abundantly found in sewage effluents undoubtedly play an ecological role in the reassimilation of nitrogen in higher forms of life.

Inorganic Growth Requirements

Oxidation of nitrite. Nitrite is rapidly metabolized by extracts of Nitrobacter with complete conversion to nitrate and the consumption of oxygen essentially equal to the theoretical amount expected according to the following reaction:

$$NO_2^- + \tfrac{1}{2}O_2 \rightleftharpoons NO_3^- \qquad (\Delta F = 17.8 \text{ kcal})$$

This oxidation provides energy in the form of adenosine triphosphate (ATP) as well as reduced pyridine nucleotides for the cellular biosynthetic reactions involving CO_2 reduction (Aleem, 1965; Kiesow, 1963, 1964). Nitrite oxidase is associatedwith the particulate constituents of the cell (Aleem and Nason, 1959). The minimal size of membrane fragments from sonic homogenates having nitrite oxidizing activity is estimated to be about 22 nm (Tsien and Laudelout, 1968), but the distribution of cytochrome a appears not to be affected below this critical size. An oxygen atom obtained from water, and not molecular oxygen, participates in the

oxidation of nitrite to nitrate (Aleem, Hoch, and Varner, 1965: Kiesow, 1964). Water may also act as a hydrogen donor for the reduction of pyridine nucleotides (Aleem, Hoch, and Varner, 1965). Nitrite oxidation is mediated by the cytochrome system and is coupled to oxidative phosphorylation (Aleem and Nason, 1959, 1960).

The theoretical P/O ratio of one has been approached by Kiesow (1964) and Aleem (1968). Aleem attributed lower P/O ratios previously obtained to inadequate conditions for growth and procedures for sonication which resulted in the destruction of the structural integrity of the phosphorylating particles without affecting the electron transport capability. When reduced nicotinamide adenine dinucleotide (NADH) served as electron donor, a P/O ratio of two was obtained.

The problem of the transport of electrons from nitrite to oxygen has been examined independently by the two groups of investigators led by Kiesow and Aleem. Aleem's studies have been with N. agilis and other chemolithotrophs, while Kiesow has limited his studies to N. winogradskyi. Both groups arrived at the concept of reversed electron flow along the electron transport chain.

Kiesow reported that under anaerobic conditions a reversal of the normal reaction of nitrite oxidation was observed in intact cells. NADH, previously found to be produced during nitrite oxidation in cell homogenates and purified fractions of submicroscopic particles, acted as

the reducing agent for the reduction of nitrate. These reactions were mediated through two cytochromes and a flavoprotein and the synthesis of ATP was coupled with the reduction of nitrate:

$$NADH_2 + NO_3^- + 2ADP + 2Pi \rightleftharpoons NAD + 2ATP + H_2O + NO_2^-$$

The same particle-fraction catalyzing the "back reaction" also catalyzed the oxidation of NADH by O_2 using a terminal oxidase:

$$NADH_2 + \tfrac{1}{2}O_2 + 3ADP + 3Pi \rightleftharpoons NAD + H_2O + 3ATP$$

Kiesow concluded that the "back reaction" occurred in reverse and that it followed the second reaction in a sequence. Since all reactants of the second reaction, except oxygen, are products of the first reaction, it was further concluded that the sequence was cyclic. The cyclic reaction therefore proceeds as follows:

$$NADH_2 + \tfrac{1}{2}O_2 + 3ADP + 3Pi \longrightarrow NAD + H_2O + 3ATP$$

$$NO_2^- + 2ATP + NAD + H_2O \longrightarrow NO_3^- + 2ADP + NADH_2 + 2Pi$$

In this scheme, nitrate serves in place of oxygen as the terminal electron acceptor. An objection to Kiesow's scheme lies in the fact that 2 moles of ATP which are required for the reduction of NAD by nitrite oxidation can not provide an energy equivalent of 34,500 calories, a potential difference of 750 mv.

The scheme proposed by Aleem's group (Aleem, 1968;

Sewell and Aleem, 1969) is thermodynamically more satisfactory. In their system, cytochrome a_1 is the site of entry of nitrite in the electron transport chain and in NAD reduction electrons are passed along the chain according to the following sequence:

$$NO_2^- \longrightarrow \text{cytochrome } a_1 \xrightarrow{ATP} \text{cytochrome c} \xrightarrow{ATP}$$

$$\text{cytochrome b } (Q_{10}) \longrightarrow \text{flavoproteins} \xrightarrow{ATP} \text{NAD}$$

The reduction of each mole of NAD by nitrite requires the utilization of approximately 5 moles of ATP. This is in agreement with the calculated 35 kcal free energy needed for the overall reverse electron flow process. The conservation of energy in the form of one ATP per nitrite oxidized is effected by the entry of nitrite at cytochrome a_1 and the subsequent reduction of oxygen by the terminal oxidase of the electron transport chain. Kiesow's scheme differs in that in his scheme electron transport from nitrite to oxygen is not coupled to ATP synthesis and nitrite oxidation consumes energy which is provided by the oxidation of NADH.

Metabolism of nitrate. The presence of a nitrate reductase in N. agilis has been reported by Straat and Nason (1965). This particulate enzyme is energy independent and catalyzes the reduction of nitrate to nitrite with reduced cytochrome c as electron donor. Cytochrome a_1 and an unidentified metal component are also implicated in the reaction. Chlorate completely inhibited nitrate reduction. This compound

is known to delay growth without affecting nitrite oxidation in growing cultures of Nitrobacter (Lees and Quastel, 1945). These findings suggest that the nitrate reduction may provide the first step in the acquisition of nutritional nitrogen. At present, nothing is known of the origin of nitrogen used in the synthesis of nitrogenous compounds in Nitrobacter. The possibility that nitrate reductase also effects the recycling of nitrite involved in nitrite oxidation has not been explored.

Fixation of carbon dioxide. In the chemolithotrophic bacteria carbon dioxide fixation is accomplished by a combination of enzymatic reactions from widely distributed pathways and specialized reactions which are found only among autotrophic organisms. The common path of carbon, shared by photolithotrophic and chemolithotrophic systems, utilizes reactions which are also part of glycolysis, pentose phosphate metabolism, and dicarboxylic acid metabolism. The two reactions which are peculiar to the autotroph are: the phosphorylation of ribulose 5-phosphate to ribulose 1,5-diphosphate (RuDP) and the subsequent carboxylation of RuDP resulting in the formation of 3-phosphoglycerate (3-PGA).

The elucidation of the path of carbon in the photosynthetic carbon reduction cycle, of which RuDP carboxylase is a part, was made possible through the radiocarbon studies of Horecker and Racker (Vishniac, Horecker, and Ochoa, 1957). The pathway as it occurs in higher plants was described by Bassham et al. (1954). Similar mechanisms exist in the

obligate and facultative autotrophic bacteria (Elsden, 1962). Early studies on crude extracts of spinach leaves (Jakoby, Brummond and Ochoa, 1956) and Thiobacillus thioparus (Santer and Vishniac, 1955) resulted in the fixation of CO_2 in the presence of RuDP with the formation of 3-PGA. The rapid incorporation of $^{14}CO_2$ into 3-PGA and sugar phosphates was also accomplished by intact cells. The fixation of CO_2 by the RuDP pathway is now known to occur in a number of other chemolithotrophic bacteria: hydrogen oxidizing bacteria (Vishniac and Trudinger, 1962), T. denitrificans (Trudinger, 1955, 1956), T. thiooxidans (Suzuki and Werkman, 1958a; Iwatsuka, Kuno, and Maruyama, 1962), Ferrobacillus ferrooxidans (Maciag and Lundren, 1964), N. agilis (Malavolta, Delwiche, and Burge, 1960; Aleem, 1965), and Nitrosomonas europaea (Delwiche, Burge, and Malavolta, 1963).

RuDP carboxylase (EC 4.1.1.f) in facultative autotrophic bacteria such as hydrogen bacteria (Hydrogenomonas species) is formed adaptively under autotrophic conditions; the highest level of activity occurring in organisms grown under strictly autotrophic conditions (Vishniac and Santer, 1957). Similar findings have been reported for Thiobacillus novellus (Vishniac and Trudinger, 1962), T. denitrificans (Kornberg, Collins, and Bigley, 1960) and Hydrogenomonas facilus (McFadden and Tu, 1965). Thus, RuDP carboxylase appears to play an important role in chemosynthesis, and is considered the critical enzyme involved in autotrophic CO_2 fixation.

The fixation of CO_2 into oxalacetate is made possible by the action of two distinct enzymes, phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.e) and PEP carboxykinase (EC 4.1.1.32). These two enzymatic reactions are readily distinguishable by reaction reversibility and nucleotide requirements. The reaction catalyzed by PEP carboxylase is irreversible, has no nucleotide requirement, and the phosphate group of PEP is released as inorganic phosphate. Unlike the preceding reaction, the reaction utilizing the carboxykinase has a specific requirement for nucleotides, is readily reversible, and widely distributed in nature. Both of these enzymes have been found simultaneously in T. thiooxidans (Suzuki and Werkman, 1957, 1958b). The existence of these carboxylating enzymes has been established for N. europaea (Delwiche, Burge, and Malavolta, 1963) and N. agilis (Aleem, 1965) but the predominant type has not been identified.

Malate dehydrogenases (EC 1.1.1.38 or 1.1.1.40) which catalyze the reversible formation of L-malate from CO_2 and pyruvate have been observed in N. agilis (Smith and Hoare, 1968). The addition of acetate to an autotrophic medium containing nitrite had no effect on the levels of enzyme in the cell extracts. A pathway for carbon assimilation involving CO_2 has been recently discovered by Evans, Buchanan and Arnon (1966) in photosynthetic bacteria. It has been described as a cyclic process in which enzymes of the TCA cycle operate in reverse order and are coupled to pyruvate synthetase, α-ketoglutarate synthase, PEP synthetase, and PEP carboxylase. The

first two reactions are the key reactions in this cycle and are ferredoxin-dependent CO_2-fixation reactions. These two CO_2-fixation reactions reverse two reactions of the TCA cycle that in aerobic cells are irreversible. Therefore, it is highly unlikely that this mechanism of CO_2-fixation occurs in Nitrobacter since ferredoxin-dependent CO_2-fixation has been demonstrated only in anaerobic bacteria.

Effect of other inorganic ions. The mineral requirements, except for the carbon and energy sources, resemble those known for heterotrophs. The nutrient demand in laboratory studies is low since the number of cells is relatively small. The optimal iron concentration for growth of N. winogradskyi is reported to be about 6 µg per ml for the oxidation of 200 µg per ml nitrite-N (Meiklejohn, 1953) but this iron level exceeds the amount of cell carbon formed. The use of well-defined culture methods has been recently initiated for nutritional studies of N. agilis (Aleem and Alexander, 1960). Using these methods and an alumina purified inorganic medium, the requirements for various ions were studied by omitting the nutrient under study and adding graded amounts to flasks in a standard series. The optimal nutrient levels thus obtained were approximately 5 µg per ml for both phosphorus amd magnesium and at least 0.005 µg per ml for iron. The optimal level of iron established previously by Meiklejohn may have been necessary to overcome the binding of iron by $CaCO_3$ present in the medium. Cell mass development and

concomitant nitrite oxidation is enhanced in freshly inoculated cultures if the medium contains small amounts of molybdenum (Finstein and Delwiche, 1965; Zavarzin, 1958). Zavarzin postulated that a molybdo-flavoprotein is concerned in the energy yielding reaction of these autotrophs. According to investigations of Finstein and Delwiche the enhancement may simply be a response in which greater cell mass occurs and not a direct molybdenum function in enzymatic nitrite oxidation. A function of molybdenum in nitrate reductase (Nicholas, Nason, and McElroy, 1953) does suggest a function in the reverse direction. In spite of early reports that Ca ion was required, Alexander (1965) believes there is no valid evidence for such a requirement. As would be expected, potassium and sulfur are required elements (Welch and Scott, 1959) and copper is reported to be stimulatory (Kiesow, 1962, Zavarzin, 1958). N. agilis is markedly inhibited as are other Nitrobacter isolates by the presence of ammonium salts (Bomeke, 1950; Boullanger and Massol, 1903; Meyerhof, 1916). In recent studies, the presence of 100 µg ammonium per ml completely prevented the growth of N. agilis and as little as 10 µg ammonium per ml delayed growth (Aleem and Alexander, 1960). In nonproliferating cell suspensions, the amount of oxygen uptake in the presence of 70 ng ammonium per ml is dependent upon pH. At pH 6.0 to 7.0 no inhibition occurs, but with increasing pH the % inhibition increases until there is an 82% inhibition at pH 9.5. The ammonium inhibition of intact cells apparently is unrelated to the nitrite oxidase

since the formation of nitrate from nitrite was unaffected when as much as 700 ng ammonium per ml was incubated with a cell extract.

Factors Limiting Growth Yields

A retardation in the initiation of growth when nitrite-N concentrations exceeded 130 µg per ml was demonstrated by Aleem and Alexander (1960). Additions of up to 500 µg per ml could be made without growth suppression once exponential growth had commenced. The maximum amount of nitrite-N that could be transformed was found to be 4000 µg per ml. The soil isolate of Gould and Lees (1960) appeared to exhibit greater sensitivity to nitrite than did N. agilis which was used in the investigations of Aleem and Alexander. Gould and Lees found that increments greater than 300 µg nitrite-N per ml added to growing cultures resulted in a temporary depression of the oxidation rate, and 600 µg nitrite-N added at one time delayed oxidation for at least one week. Oxidation and growth both ceased after 2200 µg nitrite-N had been oxidized. An important observation made by Gould and Lees was that the rate of oxidation remained logarithmic until ca. 200 µg nitrite-N per ml had been oxidized. Subsequently, the oxidation rate was linear and dependent upon the rate of air flow through the culture.

Nitrate also exerts toxic effects on these organisms. Nitrification was completely inhibited at the time of inoculation by the presence of 5000 µg nitrate-N per ml and

amounts of 1000 to 2000 µg per ml prolonged the lag phase. As much as 500 µg nitrate-N per ml had no effect on the rate of nitrite oxidation of newly inoculated cultures. Actively growing cultures are less sensitive to nitrate-N since amounts of 2000 to 5000 µg N per ml do not interfere with the process of nitrite oxidation. Gould and Lees (1960) support the theory that the accumulation of nitrate is deleterious to growth which appears to conflict with the data presented by Aleem and Alexander. Finstein and Delwiche (1965) clarified this discrepancy when they demonstrated that nitrate accumulation did not contribute to a decline in efficiency until a concentration of 0.1 M (1400 µg N/ml) was attained.

Until very recently it was not possible to propagate Nitrobacter in quantities sufficient for biochemical study. Improved culture methods were first made available by Aleem and Alexander (1958) who propagated this bacterium in 8 liters of a medium free of chemical precipitate. The cultures were grown in 10-liter serum bottles aerated with a continuous stream of sterilized air dispersed with glass spargers. Using this cultivation procedure, Ida and Alexander (1965) were able to obtain only 50 to 60 mg of dry cells per liter of medium after 6 to 7 days of growth. Gould and Lees (1960) improved the cell dry weight yield by combining nitrate removal by dialysis and intense aeration of cultures grown in a custom built, 5-liter fermentor. Under these conditions, a cell dry weight of 65 mg per liter was obtained upon the oxidation of 2000 µg of nitrite-N per ml. When nitrate removal was combined with intense aeration, a 500-ml fermentor

culture produced ca. 1.0 g dry weight per liter per 30,000 µg nitrite-N oxidized per ml of culture (within 6.5 to 7 days) without the onset of a stationary phase. In the 500-ml fermentor, there was a greater amount of oxygen supplied to the culture than in the 5-liter fermentor.

Pure Culture Methods

Considerable difficulty still exists in obtaining cultures free of contaminating microorganisms. These contaminants often remain undetected because of their morphological similarity to Nitrobacter and their incapacity to develop on conventional heterotrophic laboratory media. Common contaminants in final enrichment cultures of the nitrifiers often include species of Pseudomonas, Hydromicrobium, Mycobacterium, Flavobacterium, and Serratia as well as an occasional myxobacterium (Gundersen, 1955). A symbiotic relationship may exist between Nitrobacter and members of these species which might explain the growth of the autotroph in unfavorable soil environments. In the laboratory, the problem of contaminants is not ended once the nitrifier is isolated in pure cultureas recontamination during laboratory manipulations occurs quite readily (Garretson and San Clemente, 1967).

Metabolism of Organic Compounds

Since Winogradsky (1891) first isolated Nitrobacter in pure culture, numerous reports have appeared on the influence of organic compounds on growth and nitrification. Unfortun-

ately, the concept of nonutilization of organic compounds and indeed their inhibitory effect at relatively low concentrations which originated with the early work of Winogradsky was carried over in subsequent investigations on the utilization of organic amendments. The use of cultures of mixed flora and unrefined growth conditions has further propagated the concept of organic inhibition.

During the last decade, a number of attempts have been made to account for the failure of the nitrifiers, and other species of chemolithotrophs, to utilize organic compounds as sole carbon sources. Evidence for the lack of a permeability barrier in N. agilis was simultaneaouly presented by Ida and Alexander (1965) and Delwiche and Finstein (1965). These investigators found that acetate, glycine, hypoxanthine, and glycerol were readily incorporated into the cells. However, neither group of investigators was able to demonstrate growth of the organism on organic compounds. A lack of stimulation in nitrite oxidation was observed with a number of amino acids and vitamins, including biotin (Aleem and Alexander, 1960). Krulwich and Funk (1965) reported enhancement of both nitrite utilization and growth with biotin using four strains of N. agilis. This stimulation may be artificial since the optimal growth yields, as compared to results obtained by other investigators, were not obtained from the control cultures. A slight stimulation of nitrification and growth, as determined by optical density, was reported with yeast extract, Vitamin Free Casamino Acids, and some amino acids (Delwiche

and Finstein, 1965). Unfortunately, only optical density was used for measuring growth in the presence of all of the compounds, except yeast extract. Growth of cultures containing yeast extract resulted in an increased viable cell count. The oxidation of formate has been demonstrated in both N. agilis and N. winogradskyi (Malavolta, Delwiche, and Burge, 1962; Van Gool and Laudelout, 1966). The oxidizing enzyme bears a resemblance to formic dehydrogenase of heterotrophic bacteria in that it is cytochrome specific, particulate, and not successfully solubilized. The enzyme shows maximum activity around pH 7.0 and is stimulated by ATP. However, no growth was obtained when formate was the sole energy source (Van Gool and Laudelout, 1966) and formate contributes only a small fraction of carbon to resting cells (Delwiche and Finstein, 1965).

Absence from the cell of the appropriate enzymes to oxidize permeable organic molecules has not been established for N. agilis. Cell-free extracts are known to contain acetyl-coenzyme A (CoA) synthetase (EC 6.2.1.1.) and all of the enzymes of the TCA cycle (Aleem, 1965; Smith and Hoare, 1968).

In N. agilis, the generation of assimilatory power, production of ATP and reduced pyridine nucleotides, is coupled to the oxidation of nitrite (Aleem, 1965). A particulate NADH oxidase (EC 1.6.99.3) has also been found in crude extracts (Smith and Hoare, 1968). Thus it is certain that Nitrobacter has the assimilatory power which could be coupled to the oxidation of acetate or other organic

compounds.

Until Smith and Hoare (1968) suggested that N. agilis was a facultative autotroph, this microorganism was classified as an obligate autotroph. This classification had been previously unchallenged for over 60 years. The reasoning of Smith and Hoare was based on the ability of this organism to grow "heterotrophically through seven transfers" on a medium containing both acetate and casein hydrolysate. If their results can be confirmed, other obligate autotrophs should be examined for their growth potential under similar growth conditions. If presumptive obligate autotrophs prove to be capable of growth on organic substrates then more appropriate criteria will have to be applied in the classification of autotrophic microorganisms.

A biochemical basis of obligate autotrophy in blue-green algae and thiobacilli was proposed by Smith, London, and Stanier (1967). The absence of α-ketoglutarate dehydrogenase (EC 1.2.4.2) and NADH oxidase was reported in the obligate autotrophs T. thiooxidans and T. thioparus, but the facultative autotrophs T. intermedius and Hydrogenomonas eutropha did not possess these enzymatic deficiences. However, the presence of NADH oxidase activity has been established by other investigators in obligate autotrophs including Thiobacillus neapolitanus, T. thioparus, and T. thiooxidans (Rittenberg, 1969). Among the nitrifiers, both α-ketoglutarate dehydrogenase and NADH oxidase activity have been detected in Nitrosomonas oceanus (Williams and Watson, 1968) and N. agilis (Smith and Hoare, 1968). In N. europaea both

these enzymes are lacking (Hooper, 1969). As in the thiobacilli, nitrifiers appear to be inconsistent in respect to the activity of these enzymes, and no common basis for obligate autotrophy has been established.

The metabolic response of autotrophs to growth in a medium containing acetate may prove to be valid in characterizing differences between obligate and facultative chemoautotrophs. In the facultative chemoautotroph, T. intermedius, approximately 40% of the carbon in newly synthesized cellular material is contributed by acetate, whereas in a number of obligate autotrophs acetate contributes only 10% of the carbon in newly synthesized cellular material (Smith, London, and Stanier, 1967).

Synthesis of Storage Materials

The presence in Nitrobacter of sudanophilic granules containing poly-β-hydroxybutyrate (PHB) was first described by Tobback and Laudelout (1965). A massive accumulation of this reserve material was reported to occur within N. agilis cells cultured either in the presence of acetate and casein hydrolysate, or in the presence of acetate and limited nitrite over a prolonged period (Smith and Hoare, 1968; Pope, Hoare, and Smith, 1968). Lesser amounts of PHB were evident when growth was on a mineral medium supplemented with acetate and unlimiting nitrite.

Members of the genus Hydrogenomonas, which are facultative chemolithotrophic bacteria, accumulate PHB under conditions where energy and carbon sources are available but

growth and multiplication are limited by the lack of some additional factor, for example, nitrogen or phosphorus (Schlegel, 1968). In Hydrogenomonas, PHB is known to function as storage material for protein synthesis when the usual conditions for growth are absent. These organisms also store phosphate primarily as polymerized inorganic metaphosphate (polyphosphate, volutin). Since the amount of energy thus fixed is extremely low, the storage of polyphosphate is significant only for phosphate balance. The storage of polyphosphate in Nitrobacter has not been investigated.

Acetate is incorporated as a total unit during PHB synthesis in Hydrogenomonas. The pathway proceeds via acetyl-CoA, acetoacetyl-CoA, ß-hydroxy-butyryl-CoA, poly-ß-hydroxy-butyrate. The synthesis of PBH from CO_2 by the reductive pentose phosphate pathway gives uniformly labeled acetate and PHB. This pathway occurs via 3-PGA, 2-PGA, PEP, pyruvate, and acetyl-CoA.

Physiological Classification of Chemolithotrophs

In an excellent review Schlegel (1968) described the manner in which cells derive cell carbon and energy from organic matter in terms of our present knowledge of the chemolithotrophic soil bacteria. The terms "autotrophs" and "heterotrophs" are unequivocal and well understood to mean organisms which can synthesize their cell material from CO_2 as the main source of carbon or from preformed organic substances, respectively. However, it should be emphasized that autotrophs are those organisms which use CO_2 as the main

source of carbon and that occasionally they can grow with organic substrates is of lesser importance. The term chemolithotrophic pertains to organisms which derive metabolically useful energy from inorganic oxidations and the question of the derivation of cell carbon is irrelevant.

Rittenberg (1969) uses the term "mixotrophy" to describe a commingling of alternative modes of energy generation, or carbon assimilation, or both. A number of variations in the combinations involved in chemolithotrophic metabolism are possible and have been observed in a number of soil bacteria. Mixotrophic metabolism as seen in Desulphovibrio desulphuricans is the assimilation of organic substrate with the aid of energy obtained from an inorganic oxidation. These bacteria do not grow autotrophically and their lithotrophy is not obligatory. Another combination which occurs in Micrococcus denitrificans is the inability to produce most of its cellular carbon by CO_2-fixation because of low activity of the enzymes of the reductive pentose phosphate cycle and therefore a dependence on supplementation with organic substrates. A third method is the conversion of either carbon dioxide or organic substances to cellular carbon using energy only from the oxidation of inorganic compounds. On the basis of the findings of Smith and Hoare (1968), Rittenberg classified N. agilis as a mixotroph, which uses inorganic and organic energy sources concurrently, rather than as an obligate chemolithotroph.

Historically, the facultative autotrophs are recognized

by their ability to grow abundantly in either inorganic or organic media. In the presence of organic substrates, members of this group can either express simultaneously alternate physiologies, or the organic growth substrates have an inhibitory effect on inorganic oxidations. Smith and Hoare (1968) classified N. agilis in this group, but as suggested by Rittenberg the present day usuage of the term "facultative autotroph", as well as "obligate autotroph" should be eliminated.

Established Metabolic Pathways in Acetate Metabolism

Tricarboxylic acid cycle. The TCA cycle which is responsible for the terminal respiration in both animal tissues and many organisms is believed to operate in N. agilis as all of the enzymes are present in sufficient amounts (Smith and Hoare, 1968). One of the functions of this cycle in heterotrophic organisms is to bring about the oxidation of acetate but it cannot function on compounds more oxidized than acetate (Kornberg and Elsden, 1961). In addition to its oxidative function, the cycle plays a part in the synthesis of many intermediates, including the precursor of glutamate, α-ketoglutarate (Roberts et al., 1955). In order for synthetic processes to take place at the same time as oxidation, there must be a continual draining off of both α-ketoglutarate and the 4-carbon dicarboxylic acids. The latter compounds also serve as important precursors of many cell constituents. For growth to occur on acetate as both the

carbon and energy source, ancillary reactions are necessary for the formation of TCA cycle intermediates.

Glyoxylate bypass. The glyoxylate cycle has been widely observed in bacteria, fungi, algae, protozoa, nematodes, and plant tissues (Wegener et al., 1968). The glyoxylate cycle supports the continued operation of the TCA cycle and its net effect is the formation of 1 mole of malate from 2 moles of acetate (Kornberg and Krebs, 1957). The key enzymes whose simultaneous action produces this cyclic mechanism are isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2). Isocitrate lyase which catalyzes the reversible aldol cleavage of isocitrate to succinate and glyoxylate, has been observed in chemoautotrophic bacteria grown heterotrophically, including H. facilis (McFadden and Howes, 1962), M. denitrificans (Kornberg, Collins, and Bigley, 1960), as well as in N. agilis (Smith and Hoare, 1968). The condensation of glyoxylate with acetyl-CoA to form malate is catalyzed by malate synthase. Enzyme activity for this reaction has not been reported for N. agilis, but malate synthase is usually present in microorganisms in which isocitratase is also present (Kornberg and Elsden, 1961).

MATERIALS AND METHODS

Growth Conditions

Nitrobacter agilis (ATCC 14123), kindly provided by David Pramer (Rutgers—The State University) was maintained by continuous passage in the liquid autotrophic medium of Smith and Hoare (1968) which was supplemented with 0.15% $KHCO_3$ (w/v) and 0.03% $NaNO_2$ (w/v). The pH of the complete medium after autoclaving was *ca.* 9.0. Media, however, for experimental cultures were prepared by supplementation with various carbon and energy substrates of the liquid autotrophic medium (subsequently referred to as a basal medium). The basal medium contained 2.55 g Na_2HPO_4, 0.27 g KH_2PO_4, 20 mg $MgSO_4 \cdot 7H_2O$, 2.5 mg $CaCl_2 \cdot H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 11 mg Na2-ethylenediaminetetraacetic acid, 0.02 mg H_3BO_3, 0.10 mg $CuSO_4 \cdot 5H_2O$, 0.02 mg $MnSO_4 \cdot 2H_2O$, 0.02 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.15 mg $ZnSO_4 \cdot 7H_2O$, 0.01 mg $CoCl_2$ and deionized distilled water to 1 liter. The following additives were used: sodium acetate, 5 mM; Vitamin Free Casein Hydrolysate, 0.05% (w/v) (Nutritional Biochemical Corp.); glutamate, 1 or 2.7 mM; $KHCO_3$, 0.15% (w/v); $NaNO_3$, 100 μg N/ml; $(NH_4)_2SO_4$, 7.81 μg N/ml; and $NaNO_2$, at concentrations up to 1400 μg N/ml.

Cell growth was usually followed turbidimetrically in a Spectronic 20 colorimeter (Bausch and Lomb) at 440 nm and by chemical analysis of nitrite-N disappearance using the method of Rider and Mellon (1946). The autotrophic growth medium solidified with 1.5% (w/v) Noble Agar (Difco) was employed for viable counting by the pour plate method. The determination of ammonia was by direct nesslerization using the method of Peech and English (1944). Nitrate-N was estimated according to the procedure of Chase (1948).

Experimental shake flask cultures were initiated by a 6.25% (v/v) inoculum from autotrophically grown cells which had completely utilized ca. 1000 µg nitrite-N per ml. These cultures were incubated on a rotary shaker at 32 C in 2-liter flasks containing a final culture volume of 800 ml. Stationary cultures were inoculated with 2.5×10^4 organisms per ml and grown at room temperature in 250 ml Erlenmeyer flasks containing a final culture volume of 80 ml.

Cell suspensions utilized in the hot alcohol (75%) extraction of ^{14}C-labeled cellular intermediates were obtained from fermentor cultures. An MF-14 Microferm fermentor (New Brunswick Scientific Co., Inc.) equipped with two 14-liter capacity fermentor jars was operated at 30 C with a propeller speed of 200 to 3000 r.p.m. and an aeration rate of 2 to 6 liters of filtered air per min. The fermentor jars each contained 10 liters of medium identical, except for a nitrite-N concentration of 200 µg N per ml, to that previously described for shake flask cultures. Both $MgSO_4$

and $CaCl_2$ were autoclaved as a separate solution and combined with the remaining medium constituents which had been autoclaved in the fermentor assembly. First passage fermentor cultures were initiated by the transfer of 370 ml of shake flask culture which had oxidized 1200 µg of nitrite-N per ml. Second passage fermentor cultures were inoculated with a 25% (v/v) inoculum taken from a fermentor culture in the logarithmic growth phase. Exponential growth was maintained by the addition of 200 to 400 µg N per ml whenever the level of nitrite-N dropped below 100 µg per ml.

Culture Purity

Cell cultures were continuously monitored for heterotrophic contamination by pouring 0.5 ml of the culture over the surface of the following agar plates: Nutrient Agar (Difco), Potato Dextrose Agar (Difco), nitrite-free autotrophic medium containing 0.5% yeast extract, and nitrite-free autotrophic medium containing 0.2% glucose. The inoculated plates were incubated at room temperature for 10 days. A further check on culture purity was made by microscopic examination of cell pellets which were stained by the Gram stain. All heterotrophic contaminants except pseudomonads are readily distinguishable from Nitrobacter by this method. However, the common species of pseudomonads are easily detected by subculture on agar plates.

Preparation of Cell Suspensions and Chemical Analysis

Shake flask cultures were harvested by centrifugation at 9,500 X g for 20 min and the cell pellets rinsed twice with basal medium and once with distilled water. The rinsed cells were then suspended in small volumes of deionized distilled water. Cell cultures grown in the Microferm were terminated by cooling to 15 C and then harvesting the cells by continuous flow centrifugation (Sorvall Model SS-1 centrifuge equipped with the Szent-Gyorgyi and Blum continuous flow system). The sedimented cells were washed twice in cold basal medium and suspended in deionized distilled water. Total protein content was evaluated by digesting the cells in an equal volume of 1 M NaOH at 40 C for 2 hr, and then assaying according to the method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as standard. Poly-β-hydroxybutyrate (PHB) was extracted from *N. agilis* using the procedure of Tobback and Laudelout (1965). Approximately 600 to 800 ml of cell culture fluid from shake flask cultures of *N. agilis* containing 20 to 40 mg cell dry weight per liter were used for the extraction of PHB. The cell pellets obtained by centrifugation were washed twice with 20 mM phosphate buffer (18 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.65) and suspended in a final volume of 10 ml with cold distilled water. Between 4 to 5 ml of the washed cell suspension was treated under continuous mixing for 3 hr at 30 C with an alkaline solution of hypochlorite (Williamson and Wilkinson, 1958). The sediment obtained after centrifugation was

washed three times with distilled water and then successively washed with 95% (v/v) ethanol, acetone and ether. The residue was twice extracted with 6 ml volumes of boiling chloroform. Assays for PHB content were performed using a suitable volume of the chloroform extract. The methods of Williamson and Wilkinson were also employed for the crude isolation of the standard PHB from Bacillus megaterium strain KM cultures grown in Trypticase Soy Broth (BBL). The standard PHB was further purified and assayed as described by Law and Slepecky (1961). Eight 2-liter Erlenmeyer flask cultures containing 500 ml of medium were grown for 19 hr on a rotary shaker at 30 C. The cells were sedimented at 9,500 X g for 10 min and washed three times with 0.85% NaCl. The cells were digested at room temperature in alkaline hypochlorite for 1 hr and the PHB granules were washed successively with acetone, alcohol, and ether, and then dried under vacuum to a constant weight. The polymer was extracted with 100 ml of boiling chloroform and then filtered over celite. PHB was crystallized from 500 ml of acetone cooled to -20C and the crystals collected by filtration. A yield of 0.981 g of purified PHB was extracted from 28.84 g wet weight of B. megaterium cells.

Isotopic Labeling Procedure for Growing Cultures

The incorporation of acetate and bicarbonate by cells during growth was investigated in 2-liter Erlenmeyer flasks. The culture medium was amended before inoculation with

sodium acetate-1-^{14}C (10 μC) or sodium bicarbonate (1 μC) sterilized by passage through Millipore filters (HA grade). After all additions were made, the cultures had a final volume of 300 ml. To determine the rate of uptake of ^{14}C by N. agilis, 5 ml samples were taken at 12 hr intervals and drawn through Millipore filters (HA grade). The filters were rinsed twice with cold 50 mM acetate and twice with cold water, dried, and cemented to aluminum planchets. The amount of isotope in the cells retained on the filter was determined using a model 470 gas-flow detector (Nuclear-Chicago Corp., Des Plaines, Ill.) with an efficiency of 30%. Samples (0.1 to 0.2 ml) of the whole culture fluid or cell fractions were mixed with 15 ml of Bray's solution (1960) and counted in a Nuclear-Chicago (Mark II) liquid scintillation counter with an efficiency of approximately 75%.

Cultures which had been labeled with isotope were harvested after a minimal absorbance of 0.12 at 440 nm had been attained and prior to the complete exhaustion of nitrite-N. Washed cell pellets were prepared as described previously and then fractionated by the method of Roberts et al. (1955). The washed cell pellets obtained from ca. 235 ml of culture fluid were suspended in 4 ml of 75% (v/v) ethanol, heated for 30 min at 40 to 50 C, centrifuged, and the supernatant fluid collected as the alcohol-soluble fraction. This fraction was further extracted by the addition of equal volumes of ether and water, and then by the addition of just ether. The ether soluble fractions were pooled and retained as the alcohol-soluble-ether-soluble

fraction. The alcohol-insoluble precipitate was suspended in 4 ml of a mixture containing 2 ml of ether and 2 ml of 75% ethanol, heated for 15 min at 40 to 50 C, centrifuged, and the supernatant fluid collected as the alcohol-ether-soluble fraction. The precipitate was further extracted by adding 4 ml of 5% (v/v) trichloroacetic acid (TCA) and boiling for 30 min. The resultant supernatant fluid was designated as the hot TCA-soluble fraction. The residual material was then successively washed with 4 ml volumes of acid alcohol (3 ml of HCl added to 100 ml of 70% (v/v) ethanol) and ether. The insoluble residue was suspended in 2.2 ml of distilled water and 1 ml portions analyzed for protein or PHB content. For the isolation of PHB, carrier PHB (5 mg) was added to one of the portions and then the polymer isolated according to the method of Tobback and Laudelout (1965). Another portion of the cell residue was hydrolyzed in 6 N HCl for 15 hr at 105 C. Samples of the various fractions were added to Bray's solution, counted by the liquid scintillation method, and counts per min corrected for background and quenching using the channels ratio method.

Isotopic Labeling of Fermentor Grown Cell Suspensions

A 12.5 ml volume of washed cells suspended in deionized distilled water (at a concentration of 100.6 mg wet weight per ml) was placed in a 125 ml Erlenmeyer flask containing a double strength basal medium supplemented with 400 μg nitrite-N per ml and 10 mM sodium acetate. The diluted cell suspension was then incubated at 30 C in a gyrotory water bath

shaker (Model G 76, New Brunswick Scientific Co., Inc.) and equilibrated for 55 min prior to the addition of sodium acetate-2-^{14}C (200 μC). The addition of the labeled acetate, which was suspended in 2 ml of sterile deionized distilled water, resulted in a final cell suspension volume of 29 ml. At various time intervals a 2 ml sample was removed from the cell suspension and immediately transferred to a centrifuge tube containing 6 ml of absolute ethanol at 80 C. The alcoholic mixture was subsequently heated at 50 C for 30 min and the insoluble precipitate removed by centrifugation. The alcohol-insoluble fraction was rinsed with 2 ml of 20% (v/v) ethanol heated to 80 C and the supernatant fluid combined with the initial alcohol-soluble fraction. The alcohol-soluble extract was then evaporated to dryness at 50 C and the residue suspended in 2 ml of 20% ethanol.

Paper Chromatography

Whatman no. 1 paper (46 by 57 cm) was employed for the 2 dimensional separation of labeled cellular intermediates in the ethanol-soluble fraction of cell suspensions. For the chromatography of the organic acids and organic phosphates, the papers were prewashed by either descending flow or soaking in a trough for 30 min. Washing with 0.1 N HCl was followed by washing with distilled water. For the resolution of the organic phosphates, the above washes were preceded by a washing with 0.2% (w/v) ethylenediaminetetraacetic acid (EDTA), pH 8.5, to remove metallic ions. Each of the ethanolic extracts was applied to chromatograms

spotted with all of the amino acid standards. The chromatographic separation of the phosphorylated compounds was done in a similar manner. However, this procedure was not used with most of the organic acids as larger concentrations of the reference compounds were required for identification and certain of the acids were not well separated. The latter compounds were separated into 2 groups: one containing malate, succinate, fumarate, and pyruvate, and the other containing citrate, isocitrate, and glyoxylate. All of the ethanolic extracts were run individually with each group of standard compounds. Control chromatograms were prepared simultaneously using the same reference compounds which were located using various indicator sprays. Amino acids were identified using 0.25% (w/v) ninhydrin in acetone. Organic acids were detected by spraying an 0.5% (w/v) ethanolic solution of bromocresol green. Organic phosphates were identified primarily by spraying with an ammonium molybdate solution (Hanes and Isherwood, 1949) which was modified by the addition of 0.1% EDTA and followed by reduction of the phosphomolybdate comlexes with ultraviolet light. An aniline hydrogen phthalate spray (Block, Durrum, and Zweig, 1958) was used for the detection of ribose-5-phosphate.

Different solvent systems were employed for each of the classes of compounds investigated. For amino acids, the chromatograms were first developed with butanol-acetic acid-water and then with phenol-ammonia-water (Smith, 1960). Organic acids were separated using an ethanol-ammonia-water

solvent system followed by a butanol-acetic acid-water solvent system (Nordmann and Nordmann, 1960). The method of Tyszkiewicz (1962) which employs isobutyric acid-ammonia-EDTA for the first direction and butanol-propionic acid-water for the second direction was employed for the separation of the organic phsophates.

After the location of the various compounds had been established by chemical tests, the areas suspected of containing the isotopic label were scanned for radioactivity with a Model 108 mica end-window detector tube (Nuclear-Chicago Corp., Des Plaines, Ill.). Radioactive areas corresponding with the sites of the compounds identified on the control chromatograms were cut out within 4 X 6 cm rectangles and counted in 15 ml of a toluene scintillation solution with a liquid scintillation counter. The scintillation solution was prepared by dissolving 4 g of 2,5-diphenyl-oxazole and 0.05 g of 1,4-bis-2(5-phenyl-oxazoyl)-benzene in 1 liter of toluene.

RESULTS

Growth on Acetate Combined with Various Sources of Nitrogen

Stationary cultures of N. agilis were grown at room temperature for 21 days in the basal medium supplemented with various combinations of nitrogen and carbon substrates. Only the control autotrophic cultures contained sodium bicarbonate but carbon dioxide of atmospheric origin was available to the cultures. The results of this growth experiment (Table 1) clearly demonstrate that acetate in the absence of nitrite as an energy source cannot adequately support the growth of N. agilis. Neither glutamate nor nitrate additions to the acetate medium could maintain culture viability let alone support meaningful cell multiplication. Assays for nitrate-N content made periodically during the growth period showed no change in nitrate content. Ammonium sulfate, added at nontoxic levels to the acetate medium, maintained culture viability but a significant increase in plate count was not observed. The consumption of ammonium-N was slight but of sufficient magnitude for the maintenance of a viable cell population. Growth in flasks containing both acetate and casein hydrolysate was evident but the low cell yields offered little promise for metabolic study.

TABLE 1. Influence of various carbon and nitrogen sources on nitrification and growth of Nitrobacter agilis

Additions[a]	Nitrogen consumed (μg/ml)	Viable cell count[b]	
		(cells/ml)	(% of control)
$NaNO_2$ + $KHCO_3$	900	1.57×10^7	100
$NaNO_2$	919	4.94×10^7	315
$NaNO_2$ + CH_3COONa	886	2.67×10^7	170
CH_3COONa + Casein hydrolysate		5.34×10^5	3
CH_3COONa + $NaNO_3$	0	3.09×10^2	—[c]
CH_3COONa + $(NH_4)_2SO_4$	2	8.80×10^4	<1
CH_3COONa + Glutamate		—[d]	
CH_3COONa		1.20×10^4	—[c]

[a] Concentrations of the carbon and nitrogen sources are described in the text.

[b] All cultures were initiated with 2.5×10^4 cells per ml and then grown for a period of 21 days without forced aeration in a basal medium supplemented with carbon and nitrogen as indicated under Additions. The results are mean values of four replicate flask cultures.

[c] The number of viable cells after 21 days was reduced to a level below that contained in the culture at zero time.

TABLE 1. continued.

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[d] Plate counts were not made as there was no increase in absorbance.

A comparison of the results obtained with the autotrophic cultures in the presence and absence of bicarbonate indicated that the requirement for bicarbonate ions can be met with atmospheric carbon dioxide, even in the absence of forced aeration. Apparently the lowering of the pH of the medium to ca. 7.6 by the omission of bicarbonate also had no effect on nitrite oxidation.

Heterotrophic Growth on Acetate and Casein Hydrolysate

As indicated in Table 1, growth on a nitrite-free medium containing acetate and casein hydrolysate was barely perceptible. The small increase in cell number most likely was supported early in the growth period by nutrients and cellular intermediates carried over from the previous cell passage in an autotrophic medium. The effect on growth by the combination of acetate and casein hydrolysate, however, was exhaustively pursued in numerous experiments in both stationary and shake flask cultures. In preliminary investigations growth was consistently noted in the heterotrophic medium as evidenced by increased absorbance. Occasionally these cultures were overtly contaminated and were discarded, but in the remaining cultures, which appeared to be contaminant-free, growth rates mimicked those reported by Smith and Hoare (1968). In the latter cultures, heterotrophic contamination was not readily detectable by inoculation into either the media routinely used in testing for culture purity, or in the following media: Brain Heart Infusion (Difco), Nutrient Broth

(Difco), and agar supplemented with nitrite-free autotrophic medium containing acetate and casein hydrolysate at the same concentrations employed in shake flask culture. Frequently, growth of one to four colonies was noted on Nutrient Agar and less often on yeast extract agar. Initially, the appearance of these colonies was interpreted as arising from accidental exposure to environmental contaminants. After two successive passages of 17 and 28 days, one of these heterotrophic cultures was subcultured into the autotrophic medium. After a greatly prolonged period of 18 days nitrite oxidation was observed, indicating the survival of N. agilis in an organic medium. Subsequent microscopic investigations of cell pellets indicated the presence of gram-positive cocci in grape-like clusters in these cultures as well as in all of our stock lines. Since Staphylococcus aureus is widely used in other studies carried out in this laboratory, it is assumed to have been the contaminating organism. The growth of this contaminant on conventional media showed little resemblance to S. aureus insofar as growth rates were concerned. This phenomenon suggests the possibility of a biochemical mutation or selection having occurred during prolonged growth in association with N. agilis. Growth of this contaminant was suppressed in the autotrophic cultures, but in the medium supplemented with acetate and casein hydrolysate growth was greatly stimulated. No colonies of N. agilis were observed on any of the organic agars. This was confirmed by staining smears of representative colonies using the Gram stain. Those colonies which did appear on Nutrient Agar were made up en-

tirely of the coccal forms.

Growth studies were later renewed using a newly received culture of N. agilis which was scrutinized for contamination and found to be entirely free of heterotrophic microorganisms. Repeatedly, the inoculation of the heterotrophic medium with pure cultures of N. agilis resulted in either contaminated cultures or no growth at all even after 30 to 40 days incubation. The simultaneous inoculation into autotrophic media resulted in detectable nitrite oxidation within 4 days in all cases.

Synthesis of Poly-β-hydroxybutyrate (PHB)

Growth in the presence of acetate was evaluated at 32 C in 2-liter shake flasks containing the basal medium supplemented with acetate and nitrite and in some cases bicarbonate. Cultures were maintained for 89 to 104 hr with a continuous supply of nitrite. Prior to the induction of the stationary phase of growth, samples of these experimental cultures were analyzed for protein and PHB content (Table 2). In the absence of acetate, 0.3 to 0.4% PHB (on a dry weight basis) was obtained whereas cultures grown in the presence of acetate had polymer concentrations of 5.9 to 12.2%. The absence of cellular material which might interfere with the accuracy of the PHB assay was established by spectral analysis at wave lengths of 220 to 280 nm. The profiles obtained were identical to those of purified polymer isolated from B. megaterium (Fig. 1.).

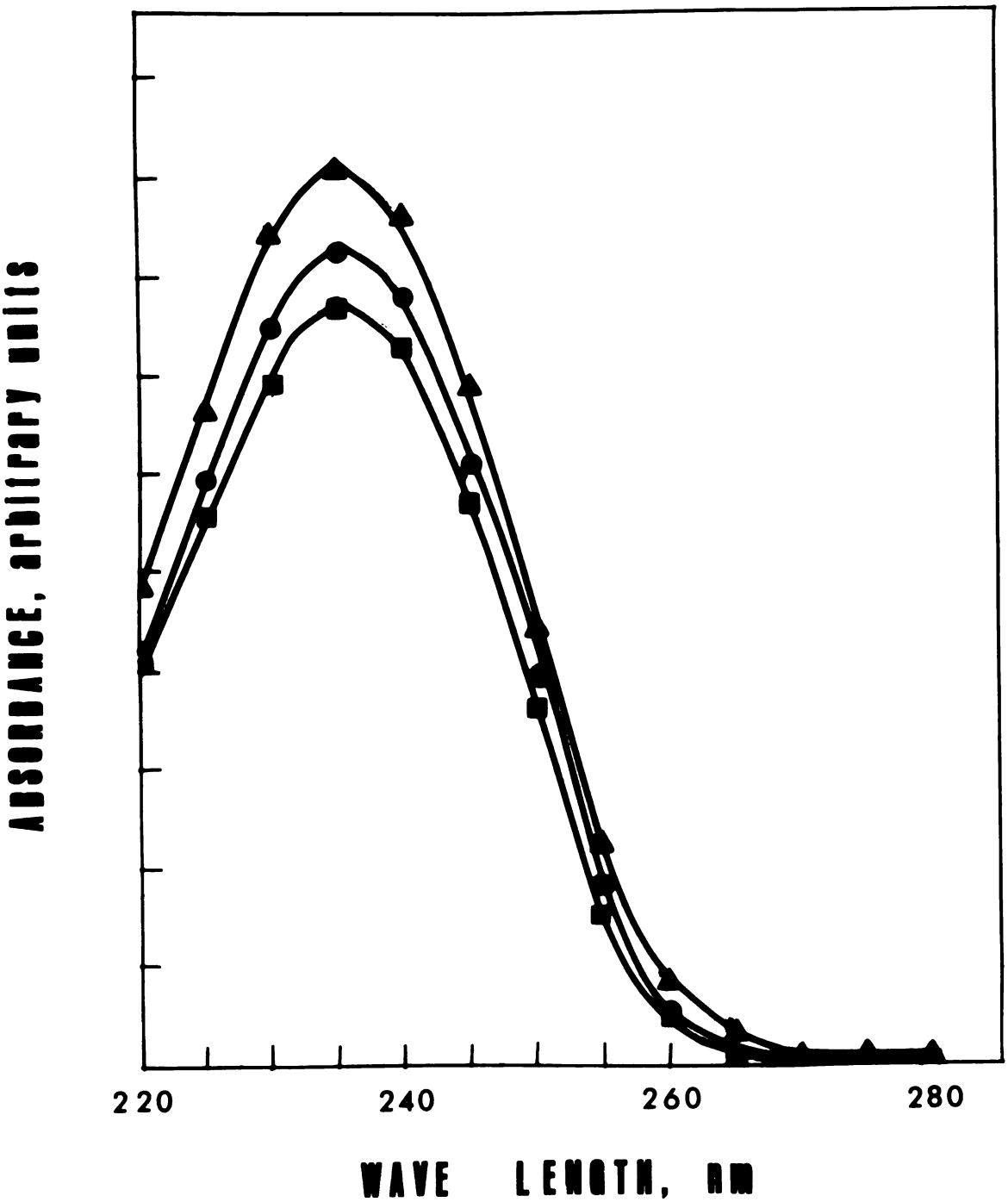
TABLE 2. Occurrence of poly-β-hydroxybutyrate (PHB) in Nitrobacter agilis grown on acetate

Nitrogen and carbon source[a]	Growth period (hr)	Nitrogen consumed (μg/ml)	Cell dry wt. (mg/L)	Total protein (%)[b]	PHB (%)[b]
$NaNO_2$ + $KHCO_3$	98	1153	30.0	36.7	< 0.3
	98	1161	29.3	39.6	< 0.4
$NaNO_2$ + CH_3COONa	89	726	23.5	24.4	12.2
	104	952	32.4	27.7	5.9
$NaNO_2$ + CH_3COONa + $KHCO_3$	89	626	19.3	19.6	7.2
	104	835	24.9	20.1	10.9

[a] Organisms were grown as shake cultures with unlimiting nitrite and cells collected prior to the stationary phase of growth. Carbon and nitrogen concentrations are cited in the text.

[b] Percent of cell dry weight.

FIG. 1. Ultraviolet absorption spectra of crotonic acid formed from poly-β-hydroxybutyrate isolated from cells of Bacillus megaterium strain KM and Nitrobacter agilis after heating in concentrated H_2SO_4. Symbols: ■, purified polymer from B. megaterium cells; ● , polymer extracted from N. agilis cells grown on basal medium plus acetate, nitrite, and bicarbonate; ▲, polymer extracted from N. agilis cells grown in the same medium lacking bicarbonate.


ABSORBANCE, ARBITRARY UNITS
220
240
260
280
WAVE LENGTH, nm

Effect of Acetate on Macromolecular Synthesis

From calculations made using the data of Table 2, it was possible to identify various relationships existing between macromolecular synthesis, culture density, and nitrite oxidized (Table 3). It was immediately evident that the synthesis of PHB during nitrite oxidation was not directly correlated to increased cell density as had been suggested by previous investigators (Smith and Hoare, 1968). The addition of acetate to the medium stimulated PHB synthesis greater than 10-fold but decreased the absorbance to dry weight ratios. If the presence of this lipid polymer were solely responsible for greater optical density, then cells containing the larger amounts of PHB would have had higher A_{440} nm per dry weight value . However, this was not the case as higher absorbance readings were obtained with the autotrophically grown cells containing the least amount of PHB and the most protein.

In order to gain insight into the effect of acetate on cell physiology, nitrite oxidation was compared with the production of cell mass and protein. On a dry weight basis, cultures containing acetate oxidized less nitrite than did the autotrophic cultures. However, on the basis of protein synthesized per nitrite oxidized, the values were higher for both the autotrophic cultures and the acetate cultures lacking bicarbonate than for the cultures containing the combination of acetate plus bicarbonate. It is concluded that less of the energy made available through nitrite oxi-

TABLE 3. Relationship of absorbance and nitrite oxidation to dry weight and protein content[a]

Nitrogen and carbon source	$A_{440\ nm}$[b]	$A_{440\ nm}$[b] / dry wt.[c]	μg Dry wt. / μg NO_2-N oxid.	ng Protein / μg NO_2-N oxid.
$NaNO_2$ + $KHCO_3$	208	6.93	0.026	9.54
	204	6.96	0.025	9.99
$NaNO_2$ + CH_3COONa	164	6.98	0.032	7.91
	181	5.59	0.034	9.41
$NaNO_2$ + CH_3COONa + $KHCO_3$	105	5.44	0.031	6.05
	131	5.26	0.030	6.00

[a] Cultures were those described in Table 2.

[b] Absorbance observed at 440 nm and multiplied by 10^3.

[c] Dry weight in mg per liter.

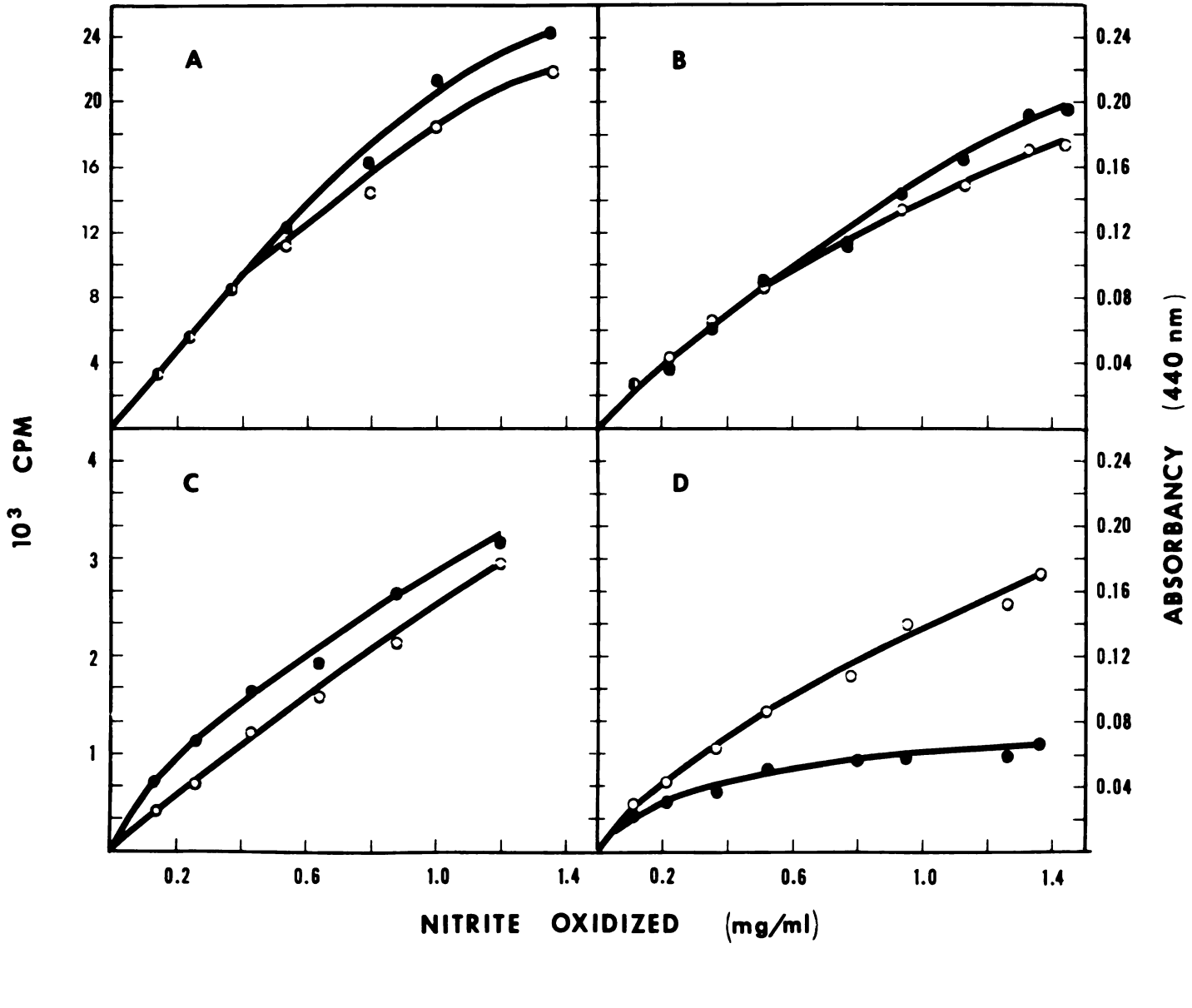
dation is coupled with the mechanism for protein synthesis during growth in a medium containing both acetate and bicarbonate.

Uptake of Isotopically Labeled Acetate and Bicarbonate by Growing Cultures

The rates of incorporation of acetate-1-^{14}C (7.74×10^4 dpm per ml) and sodium bicarbonate (7.79×10^3 dpm per ml) were followed in 2-liter shake flasks containing a final culture volume of 300 ml. Except for the addition of the labeled substrates and a reduced culture volume, growth conditions were identical to those of the previous experiment. A duplicate series of cultures in which isotopes were omitted was included in this study for following nitrite oxidation and absorbance changes during the course of this experiment. The use of duplicate cultures minimized the frequency and amount of sampling of the ^{14}C-containing cultures. The uptake of ^{14}C and absorbance are plotted against nitrite oxidation in Fig. 2.

As in the previous experiment, the addition of bicarbonate to cells growing on acetate resulted in a decrease in growth rate per unit of nitrite oxidized. This was evident by comparison of the rates of increase in absorbance shown in Fig. 2B and 2D with 2 A. The rates of uptake of acetate-1-^{14}C approximated the absorbance rates. In addition, unlabeled bicarbonate does not appear to compete with labeled acetate for entry into the cells. That acetate competes with labeled $NaH^{14}CO_3$ for uptake by N. agilis is shown in Fig. 2C

FIG. 2. Absorbance (o) and uptake of ^{14}C-labeled acetate or bicarbonate (●) in Nitrobacter agilis growing in a mineral medium containing sodium nitrite. (A) acetate-1-^{14}C. (B) acetate-1-^{14}C and unlabeled bicarbonate. (C) bicarbonate-^{14}C. (D) bicarbonate-^{14}C and unlabeled acetate.


A
B
C
D
24
20
16
12
8
4
4
3
2
1
0.24
0.20
0.16
0.12
0.08
0.04
0.2
0.6
1.0
1.4
10^3 CPM
ABSORBANCY (440 nm)
NITRITE OXIDIZED (mg/ml)

and 2D.

Incorporation of $CH_3{}^{14}COONa$ and $NaH^{14}CO_3$ into Cellular Fractions of Growing Cultures

The addition of unlabeled bicarbonate to cultures containing acetate-1-^{14}C resulted in a 25.1 % reduction in the total radioactivity (disintegrations per min per ug cell dry wt.) recovered in all of the combined fractions (Table 4). Therefore, it appears that approximately 25% of the radioactive carbons incorporated from acetate-1-^{14}C were replaced by unlabeled carbon atoms. However, when unlabeled acetate competed with labeled bicarbonate, a calculated reduction in radioactivity of 80% was attributed to the replacement of labeled carbon atoms with unlabeled carbon.

The percent distributions of ^{14}C among cellular fractions from cultures grown on labeled acetate in the presence and absence of bicarbonate were also compared with each other and those of autotrophic cultures grown on labeled bicarbonate. The distribution pattern obtained with ^{14}C-acetate as a tracer was little affected by the presence of unlabeled bicarbonate. When the contribution of PHB was omitted in the recalculation of the percent distributions, the percentages of both the alcohol and protein fractions of the two cultures differed from their former values by only 2%. The values for the alcohol-soluble fractions were increased to 23.6% for the acetate culture and to 21.6% for the acetate plus bicarbonate culture, while the protein fractions inincreased to 55.4 and 58.4%, respectively. When these

TABLE 4. Percentage distribution of ^{14}C derived from $CH_3{}^{14}COONa$ and $NaH^{14}CO_3$ among cellular fractions

Fraction	Carbon substrate[a]							
	$CH_3{}^{14}COONa$		$CH_3{}^{14}COONa + NaHCO_3$		$NaH^{14}CO_3$		$NaH^{14}CO_3 + CH_3COONa$	
	dpm[b]	%	dpm	%	dpm	%	dpm	%
Alcohol-soluble	20.7	16.9	13.5	14.6	5.8	23.4	1.0	20.7
Alcohol-ether-soluble	2.6	2.1	1.8	1.1	0.5	2.1	0.1	2.5
Hot-TCA-soluble	10.5	8.5	6.8	7.4	5.9	23.5	1.2	23.1
Acid alcohol wash	3.8	3.1	2.6	2.8	1.0	4.1	0.3	5.8
Ether wash	1.5	1.2	1.1	1.2	0.1	0.3	0	0
Insoluble residue[c]	84.2	68.2	66.2	72.0	11.6	46.2	2.4	47.9
Poly-R-hydroxybutyrate	35.6	28.7	30.1	32.8	0.3	1.1	<0.1	0.8
Protein	48.5	39.6	36.1	39.3	11.3	45.6	2.4	47.1
Total	123.3	100.0	92.3	100.0	24.9	100.0	5.0	100.0

TABLE 4. continued.

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[a] Flask cultures all contained nitrite. Carbon substrates were added as described in the text.

[b] Values are expressed as disintegrations per min per μg of cell dry weight.

[c] Poly-β- hydroxybutyrate and protein were isolated from the insoluble residue.

values were compared with those obtained from cultures provided with labeled bicarbonate, the percent distributions for all four cultures differed only in respect to the hot-TCA-soluble and protein fraction. Approximately 10% less radioactivity was observed in the protein fractions from the bicarbonate labeled cultures, than in the acetate labeled cultures. However, the hot-TCA-soluble fraction from the bicarbonate labeled cultures contained twice the amount of isotope.

Uptake of $^{14}CH_3COONa$ by Cell Suspensions

Washed cell suspensions which were preadapted to growth on acetate during a second passage in fermentor cultures (Fig. 3) were utilized for the incorporation of acetate-2-^{14}C into ethanol-soluble fractions. Adaptation to growth in a medium containing acetate and no bicarbonate required a transition period between first passage autotrophically grown cultures and acetate-adapted cultures. The generation time for the transition period was increased from 27.5 hr (generation time of autotrophic culture) to 65.0 hr. Once the cell culture became adapted to growth in the presence of acetate the generation time was reduced to 35.0 hr.

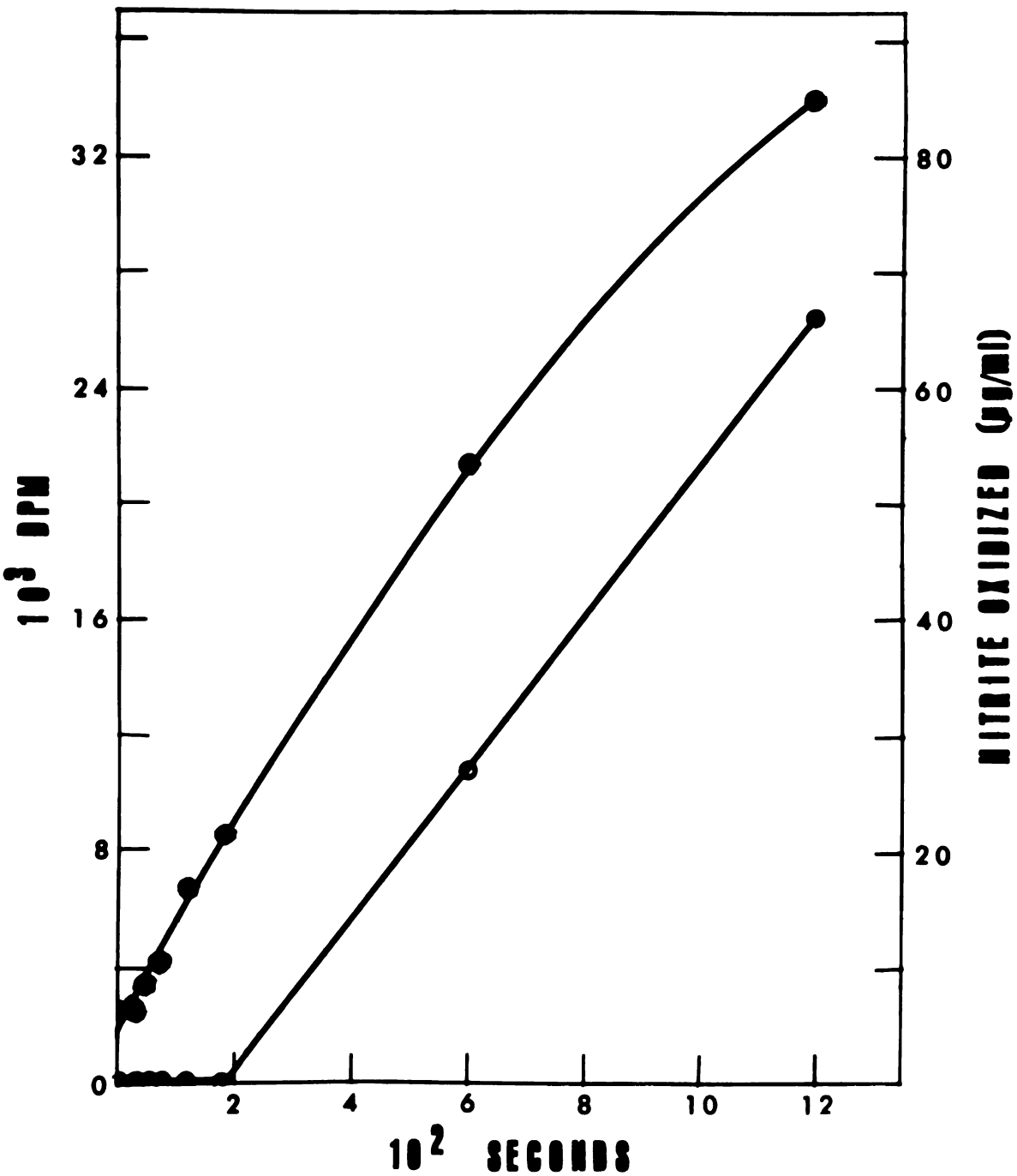
The washed cell suspension which was preincubated with nitrite for 55 min before the addition of acetate-2-^{14}C continued to oxidize nitrite at a rate linear with time until the nitrite-N was exhausted at 1200 sec (Fig. 4). The rate of incorporation of isotope into the ethanol-soluble fraction of the cells slowly decreased after 200 sec, but the

FIG. 3. Comparison of growth of Nitrobacter agilis on acetate and nitrite with growth on bicarbonate and nitrite in fermentor cultures in terms of absorbance at 440 nm and nitrite oxidation (mg oxidized per ml). Curves A and D, growth of a first passage culture in the basal medium plus nitrite and bicarbonate; curves B and E, growth of a first passage culture in the basal medium plus acetate and nitrite; curves C and F, growth of a second passage culture in the basal medium plus acetate and nitrite. The logarithms of the measured values are transposed to make the curves coincide and the distance between horizontal lines corresponds to one doubling. The vertical lines indicate time zero for the individual curves.

LOG ABSORBANCE AT 440nm
D
E
F
50
150
250
350
450
HOURS

LOG NITRITE OXIDIZED
A
B
C
50
150
250
350
450
HOURS

FIG. 4. Rates of nitrite oxidation (o) and incorporation of ^{14}C (●) into a group of selected cellular intermediates present in the ethanol-soluble fraction of a cell suspension of Nitrobacter agilis growing on acetate-2-^{14}C. The incorporation of ^{14}C is expressed as disintegrations per min per 0.05 ml of the culture and represent the sum total of radioactivity detected chromatographically in the various cellular intermediates studied.


32
24
16
8
0
10^3 DPM
80
60
40
20
NITRITE OXIDIZED (μg/ml)
2
4
6
8
10
12
10^2 SECONDS

isotope continued to be incorporated for the duration of the experiment (210 min). During the course of this experiment the protein concentration increased from 2.07 mg per ml to 3.0 mg per ml of cell suspension.

Distribution of Radioactivity in Cellular Intermediates During Nitrite Oxidation

Within 5 sec radioactiviy was detected in many of the intermediates studies. Particularly high amounts were located in the carboxylic acids of the TCA cycle and in glutamate directly derived by the amination of α-ketoglutarate (Tables 5 and 6). The compounds predominately labeled initially were citrate plus isocitrate and succinate plus fumarate. These same compounds retained the greatest amount of label for the first 180 sec. Since the TCA cycle undoubtedly functions in N. agilis (Smith and Hoare, 1968) when acetate is present in the medium, the high amounts of incorporation of label into citrate indicates that acetate was incorporated into the TCA cycle by condensation with oxalacetate. Since malate had relatively little label initially, there was no evidence for additional incorporation of acetate into malate as would have been the case had the glyoxylate bypass been operative. Among the phosphorylated compounds studied, phosphoenolpyruvate (PEP) and adenosine monophosphate were the most highly labeled compounds initially (Table 7). The label accumulated more slowly in 3-PGA than in PEP which may have been derived

TABLE 5. Incorporation of the ^{14}C of acetate-2^{14}C into organic acids of the ethanol-soluble fractions of a growing cell suspension of Nitrobacter agilis

Time (sec)	Radioactivity[a]				
	Citrate and isocitrate	Fumarate and succinate	Malate	Pyruvate	Glyoxylate
5	679	966	68	232	99
35	956	677	72	136	53
50	1,129	1,194	69	127	166
80	1,034	1.221	87	549	129
120	2,600	1.446	116	937	3
180	1,948	2,025	193	1,481	8
600	1,936	3,858	482	845	4,068
1,200	2,424	4,836	1,209	1,833	7,079

[a] All values represent disintegrations per min per 0.05 ml of cell suspension.

TABLE 6. Incorporation of the ^{14}C of acetate-2-^{14}C into amino acids of the ethanol-soluble fractions of a growing cell suspension of Nitrobacter agilis

Time (sec)	Radioactivity[a]						
	Glu	Pro	Ala	Leu	Asp	Lys	Gly
5	76	13	19	16	17	8	7
35	120	11	14	16	17	10	6
50	263	12	24	17	23	3	9
80	369	5	48	23	20	5	16
120	739	11	24	16	28	9	11
180	1,267	23	52	24	37	10	10
600	6,329	266	71	43	118	20	39
1,200	9,514	884	148	44	125	38	70

[a] All values represent disintegrations per min per 0.05 ml of cell suspension. Abbreviations for the amino acids are: Glu, glutamate; Pro, proline; Ala, alanine; Leu, leucine; Asp, aspartate; Lys, lysine; Gly, glycine.

TABLE 7. Incorporation of the ^{14}C of acetate-2-^{14}C into phosphorylated compounds of the ethanol-soluble fractions of a growing cell suspension of Nitrobacter agilis

Time (sec)	Radioactivity[a]						
	R-1,5-P	3-PGA	PEP	AMP	ADP	ATP	SPM
5	15	10	42	51	18	10	11
35	15	9	62	71	13	7	13
50	15	11	56	79	10	10	19
80	22	16	54	100	7	10	16
120	41	39	131	78	11	16	49
180	39	67	113	65	15	25	52
600	135	95	304	86	23	46	413
1,200	413	176	310	132	47	94	906

[a] All values represent disintegrations per min per 0.05 ml of cell suspension. Abbreviations for the phosphorylated compounds are: R-1,5-P, ribulose-1,5-phosphate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine tri-

TABLE 7. continued.

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phosphate; SPM, sugar phosphate mixture which includes hexose mono- and di-phosphates, sedoheptulose-7-phosphate, and ribose-5-phosphate.

from oxalacetate not synthesized by the autotrophic CO_2-fixation mechanism. Aspartate which has been shown to be labeled initially to a greater extent than glutamate during both the fixation of $H^{14}CO_3$ (Aleem, 1965) and the metabolism of acetate by the glyoxylate cycle (Kornberg, 1958) was poorly labeled throughout the growth period in our investigation.

Curves obtained by plotting the percentage of the total radioactivity isolated chromatographically from each samle against time indicate the variation in the distribution of label from acetate-2-^{14}C incorporated into the various classes of cellular intermediates present in the ethanol-soluble fraction. Early labeling occurred in the carboxylic acids (Fig. 5). For the first 120 sec, 73 to 82% of the label was located in the carboxylic acids, whereas the amino acids contained 6 to 12%, and the phosphorylated compounds contained 10 to 18%. During the steady state (600 to 1200 sec) in which nitrite continued to be oxidized linearly, the radioactivity associated with citrate plus isocitrate and succinate plus fumarate declined to 7 and 14% respectively (Fig. 6). However, steady state values for the organic acids (Fig. 5) were maintained by increased concentrations of glyoxylate and pyruvate. With the exception of citrate, isocitrate, glyoxylate, and pyruvate, the authentic compounds were chromatographically well separated. The chromatographic areas designated as glyoxylate and pyruvate overlapped each other as well as the citrate plus isocitrate areas. The ethanol extracts were run

570'

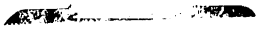


FIG. 5. The percentage distribution of ^{14}C of acetate-2-^{14}C incorporated into carboxylic acids (▲), amino acids (●), and phosphorylated compounds (o) found in the ethanol-soluble fractions of a growing cell suspension of Nitrobacter agilis. The data are expressed as the % of the total radioactivity (disintegrations per min) isolated chromatographically.

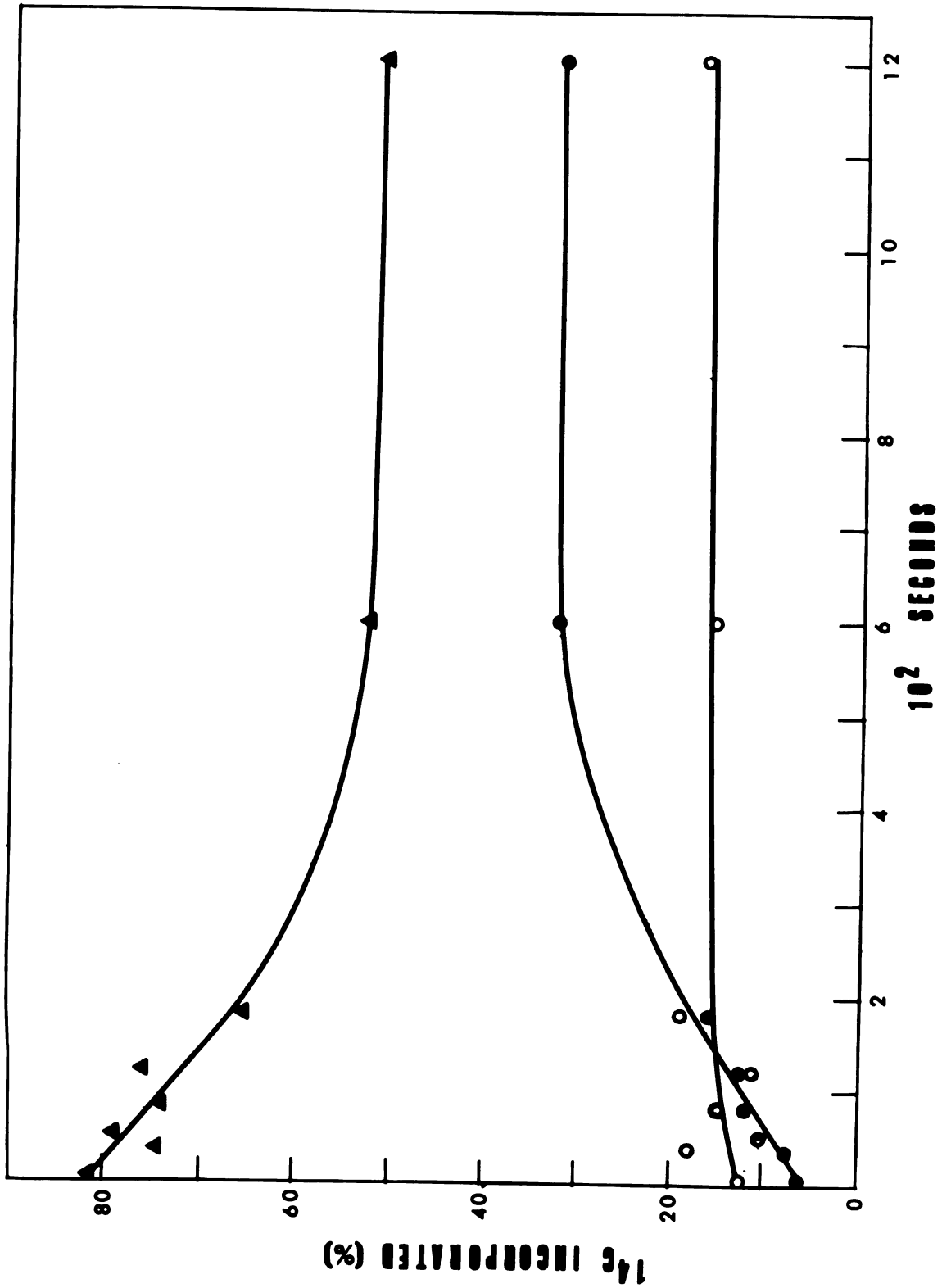
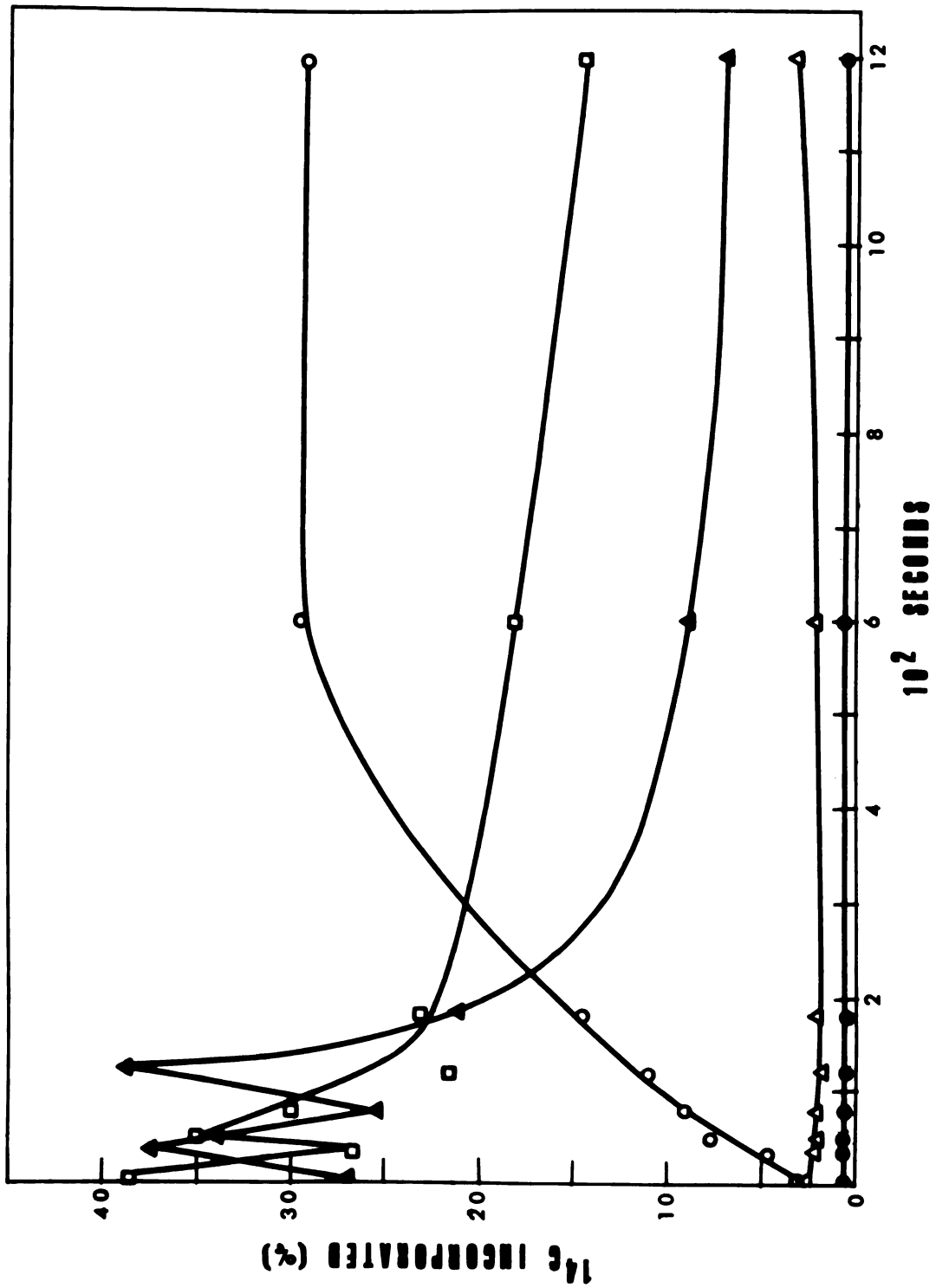
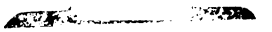

14C INCORPORATED (%)
0
20
40
60
80
2
4
6
8
10
12
10² SECONDS

FIG. 6. The percentage distribution of ^{14}C of acetate-2-^{14}C incorporated into various compounds associated with the tricarboxylic acid cycle found in the ethanol-soluble fractions of a growing cell suspension of Nitrobacter agilis. The data are expressed as % of the total radioactivity (disintegrations per min) isolated chromatographically. Symbols: o, glutamate; ●, aspartate; △, malate; ▲, citrate and isocitrate; □, succinate and fumarate.

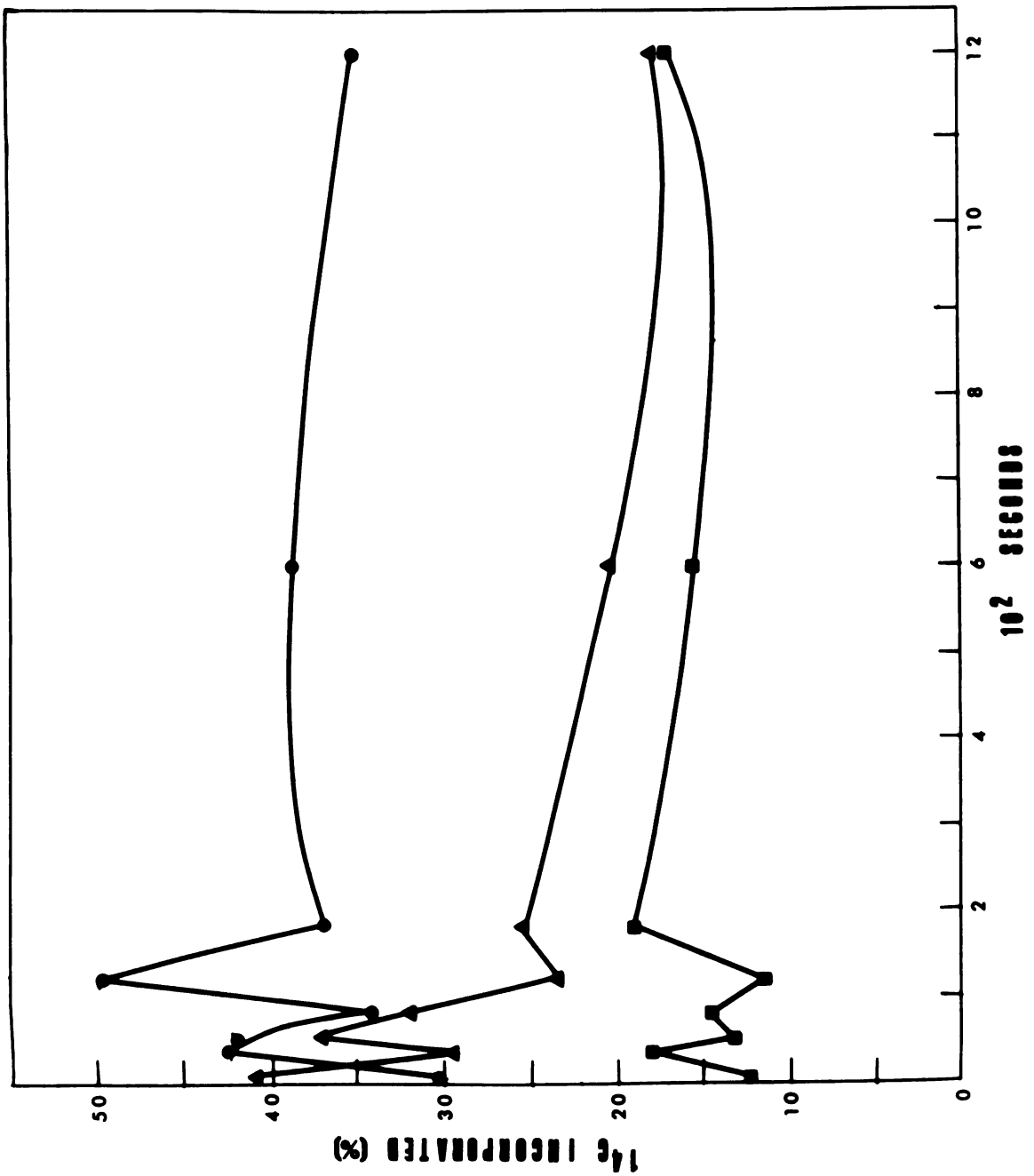

^{14}C INCORPORATED (%)
0
10
20
30
40
2
4
6
8
10
12
10^2 SECONDS



on separate sets of chromatograms, one set was cochromatographed with authentic citrate, isocitrate, and glyoxylate, while a second set was cochromatographed with authentic pyruvate and the remaining organic acids. After the isocitrate plus citrate area was cut out, the remaining activity located adjacent to this area was designated as glyoxylate. Therefore, some of the activity attributable to isocitrate plus citrate probably also contained glyoxylate, or pyruvate, or both. This mixed activity was certainly the case for the values obtained for the 120 and 180 sec samples.

After the establishment of steady state conditions, the radioactivity associated with the C_5 amd C_6 organic acids (citrate, isocitrate, and glutamate) represented approximately 35 to 38% of the total radioactivity. On the other hand, the activity of the C_4 carboxylic acids (fumarate, succinate, and malate) contained only 18 to 26% of the label (Fig. 7). Approximately 12 to 17% of the radioactivity was accounted for in the phosphorylated compounds which were formed, either by the complete oxidation of acetate to CO_2 followed by the assimilation of the released CO_2, or by the conversion of oxalacetate to PEP. The reduction of CO_2 by the autotrophic mechanism would result in the labeling of RuDP and 3-PGA, as well as the other sugar phosphates involved in the interconversion of sugars. These compounds did become labeled after relatively high levels of radioactivity were reached in the carboxylic acids.

FIG. 7. The percentage distribution of ^{14}C of acetate-2-^{14}C incorporated into phosphorylated compounds (■), C_4 dicarboxylic acids (succinate, fumarate, and malate) (▲), and C_5 and C_6 carboxylic acids (citrate, isocitrate, and glutamate) (●) found in the ethanol-soluble fractions of a growing cell suspension of Nitrobacter agilis. The data are expressed as the % of the total radioactivity (disintegrations per min) isolated chromatographically.


^{14}C INCORPORATED (%)
50
40
30
20
10
0
2
4
6
8
10
12
10^2 SECONDS

Distribution of Radioactivity in Cellular Intermediates After Nitrite Depletion

After 75 min of incubation at 30 C, the cell suspension had exhausted all of the nitrite-N in the medium. At this time the cells had been exposed to acetate-2-^{14}C for 20 min. At the 10 min sampling only 27 µg nitrite-N per ml remained, an amount which was probably inadequate for the continued generation of energy. Three additional samples were then taken at 30, 60, and 210 min and the ethanolic extracts treated as before. The cells contined to incorporate ^{14}C from acetate-2-^{14}C (Table 8). However, the percentage distribution patterns were altered considerably for many of the metabolites. The relationship between the phosphorylated compounds and the carboxylic acids formerly existing during the nitrite-oxidizing period(Fig. 7) showed a reversal after nitrite depletion (Fig. 8). The C_4 and the C_5 plus C_6 groups of carboxylic acids, together with the phosphorylated compounds represented 66 to 73% of the total label isolated. The percentage of ^{14}C appearing in the phosphorylated compounds increased concomitantly with decreases in the C_5 and C_6 acids. Interestingly, the C_4 acids remained at a fairly constant level. For the most part, the increased radioactivity among the phosphorylated compounds was attributable to increased incorporation into 3-PGA, the various sugars present in the "sugar phosphate mixture", and two unknown areas which migrated either with orthophosphate or slightly ahead of the orthophosphate area.

TABLE 8. Incorporation of the ^{14}C of acetate-2-^{14}C into various cellular intermediates located in the ethanol-soluble fractions of a cell suspension of Nitrobacter agilis deprived of nitrite-N[a]

Compound	Time (min)			
	20	30	60	210
	Radioactivity[a]			
Citrate + isocitrate	2,424	3,854	3,308	2,237
Fumarate + succinate	4,836	6,090	7,604	10,241
Malate	1,209	3,768	8,320	8,990
Pyruvate	1,833	3,756	4,512	4,325
Glyoxylate	7,079	7,798	13,786	16,268
Glutamate	9,514	11,440	15,397	18,207
Proline	884	1,623	5,178	4,120
Alanine	148	148	386	1,106
Leucine	44	65	175	707
Aspartate	125	133	308	978

TABLE 8. continued.

Compound	Time (min)			
	20	30	60	210
	Radioactivity[a]			
Lysine	38	76	234	282
Glycine	70	98	180	193
Ribulose-1,5-phosphate	413	1,060	1,510	3,099
3-Phosphoglycerate	176	695	950	9,041
Phosphoenolpyruvate	310	203	533	590
Adenosine monophosphate	132	360	645	593
Adenosine diphosphate	47	69	176	336
Adenosine triphosphate	94	139	348	924
Sugar phosphate mixture[c]	906	1,257	2,561	8,076

[a] Nitrite-N was exhausted 20 min after addition of acetate-2-^{14}C.

[b] All values represent disintegrations per min per 0.05 ml of cell suspension.

TABLE 8. continued.

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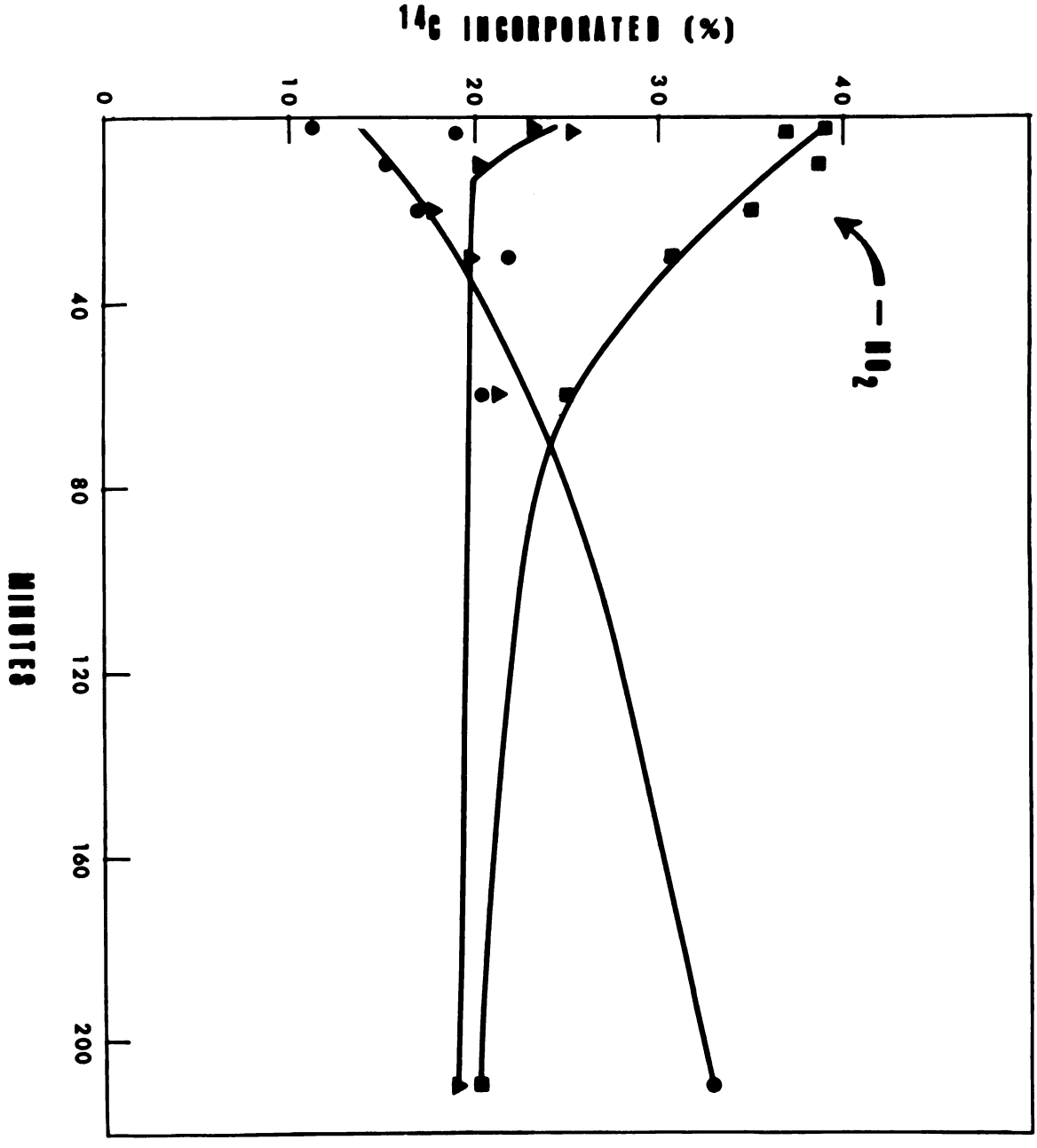
[c] The sugar phosphate mixture includes hexose mono- and diphosphates, sedoheptulose-7-phosphate, and ribose-5-phosphate.

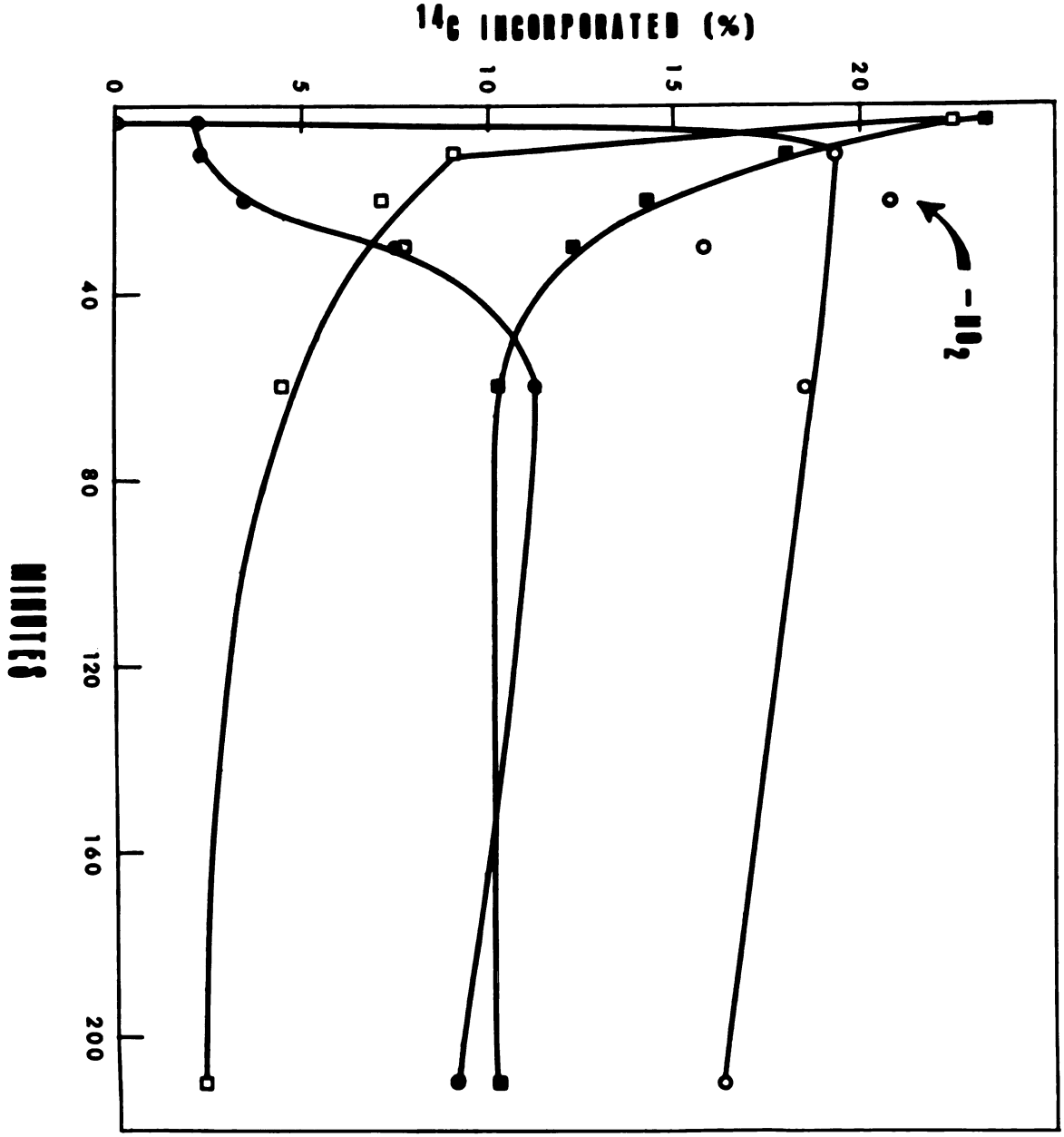
FIG. 8. The effect of nitrite depletion on the incorporation of ^{14}C from acetate-2-^{14}C into phosphorylated compounds and carboxylic acids of the tricarboxylic acid cycle. Symbols: ▲, C_4 dicarboxylic acids (succinate, fumarate, and malate); ■, C_5 and C_6 carboxylic acids (citrate, isocitrate, and glutamate); ●, phosphorylated compounds. The data are expressed as the % of the total radioactivity (disintegrations per min) isolated chromatographically.


^{14}C INCORPORATED (%)
0
10
20
30
40
MINUTES
40
80
120
160
200
H_2

The percentage levels of the carboxylic acids was also altered (Fig. 9). The radioactivity in the combinations, succinate plus fumarate and citrate plus isocitrate, progressively decreased, while malate showed an upward trend. The increased percentage levels of malate were too high to merely reflect a lag in the passage of label through the TCA cycle. Fumarate plus succinate contained similarly high amounts of radioactivity. Glyoxylate concentrations rose sharply and quickly surpassed the levels of the acids in the TCA cycle. Although glyoxylate was poorly resolved chromatographically, it was expected that the amount of isotope actually present would have been underestimated as some of its activity may have been ascribed to citrate plus isocitrate.

The overall trend of an initial lowering in isotope incorporated into components of the TCA cycle and a slower decrease in labeling in both the phosphorylated compounds and in the amino acids proline and aspartate, which are derived from the TCA cycle may indicate a cessation of the introduction of acetate into the TCA cycle at citrate after nitrite depletion. However, the sudden attainment of high levels of glyoxylate particularly after nitrite exhaustion is not compatible with the concept that acetate utilization is limited merely to oxidation via the TCA cycle and the synthesis of PHB.

FIG. 9. The effect of nitrite depletion on the incorporation of ^{14}C from acetate-2-^{14}C into glyoxylate (o), malate (●), citrate plus isocitrate (□), and succinate plus fumarate (■). The data are expressed as the % of the total radioactivity (disintegrations per min) isolated chromatographically.


^{14}C INCORPORATED (%)
0
5
10
15
20
MINUTES
40
80
120
160
200
H_2

DISCUSSION

The data reported in this paper confirm the earlier claims of previous investigators (Delwiche and Finstein, 1965; Ida and Alexander, 1965) that N. agilis, when actively replicating, relies upon nitrite as the sole source of energy and upon carbon dioxide as the principal source of carbon. The inability of acetate to serve as an energy source for the growth of N. agilis was first cited by Ida and Alexander (1965). We extended their investigations on the use of acetate, but added CO_2 and all forms of nitrogen which are usually utilized by bacteria for protein synthesis. A nitrite-free basal salts medium amended with other forms of nitrogen together with acetate and ambient CO_2 did not support meaningful growth of pure cultures of N. agilis. Clearly the utilization of acetate did not generate sufficient energy for significant cell replication.

The synthesis of PHB from acetate would appear to be of ecological value. The PHB stored under conditions where energy and substrates are readily available could be oxidized when environmental conditions become adverse. Approximately one-third of the label appearing in the insoluble residue fraction of growing cells was in PBH. However, it is concluded that during nitrite oxidation the incorporation

of acetate into protein plays the major role in the utilization of acetate.

Since both the oxidation of acetate and the presence of acetyl-CoA synthetase and all of the enzymes of the TCA cycle have been reported for N. agilis (Smith and Hoare, 1968), the labeling observed not only in the intermediates of the TCA cycle, but in the phosphorylated compounds during nitrite oxidation could have been initiated solely by this oxidative process. However, in order for growth to occur using acetate as a carbon source, acetate would have to be metabolized by some biosynthetic pathway such as the glyoxylate bypass or the glycerate pathway.

The percentage distribution patterns of ^{14}C from acetate-2-^{14}C incorporated into components of the ethanol-soluble fraction of Pseudomonas and other microorganisms which possess a glyoxylate cycle (Kornberg, 1958; Kornberg and Elsden, 1961) differ markedly from the patterns observed in N. agilis. The most significant difference is in the incorporation of ^{14}C into malate. In microorganisms having a functional glyoxylate cycle, malate initially bears more ^{14}C than does citrate plus isocitrate, or glutamate, since in the glyoxylate cycle two molecules of labeled acetate, rather than just one, contribute to the formation of malate. For the reasons just cited, a glyoxylate cycle in the presence of nitrite oxidation is believed to be nonfunctional in N. agilis. The pathways in the utilization of acetate for PHB synthesis and protein synthesis via the TCA cycle

and the glyoxylate bypass are presented in Fig. 10.

In microorganisms having an oxidative dicarboxylic acid cycle, malate again is the predominantly labeled component of the early ethanolic fractions (Kornberg and Sadler, 1961). Therefore, the dicarboxylic acid cycle also appears to be nonfunctional in N. agilis when nitrite oxidation takes place.

It is evident from the changes in the percentage distribution patterns which occurred at the time of nitrite depletion that the mode of incorporation of ^{14}C was also altered. Since the data shows an accumulation of glyoxylate just prior to and after nitrite-N was depleted and Smith and Hoare (1968) reported a considerably increased level of isocitrate lyase (100-fold) when acetate was present in the medium, it is concluded that isocitrate is split to form glyoxylate in N. agilis. The metabolism of glyoxylate initially may be prevented by low activity of malate synthase. The presence of glyoxylate in sufficient quantity may be necessary to activate enzyme formation or activity. Although malate synthase has not been found in extracts of N. agilis grown in an autotrophic medium containing both nitrite and acetate (Smith and Hoare, 1968), the absence of nitrite oxidation may be required for enzyme activation. This possibility is worthy of future investigation. The increased levels of malate and succinate in conjunction with decreased levels of citrate and isocitrate offer evidence that glyoxylate is metabolized in the absence of nitrite oxidation.

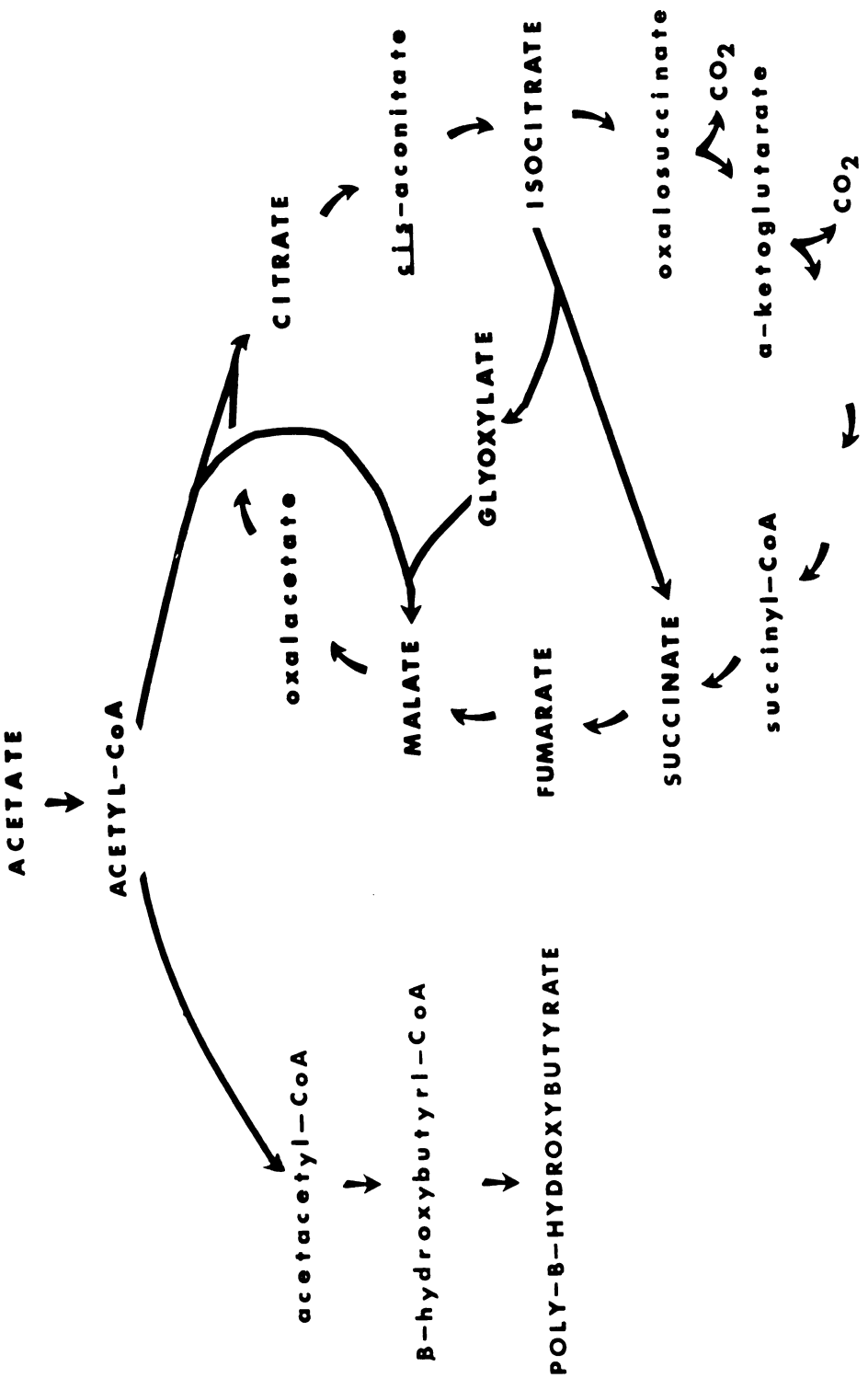


FIG. 10. Possible pathways of acetate metabolism in Nitrobacter agilis.

The data obtained from the investigation on the growth of pure cultures of N. agilis in media containing acetate does not support the reclassification of this microorganism as a "facultative autotroph" as proposed by Smith and Hoare (1968). While the isotopic kinetic studies indicate that acetate is metabolized via glyoxylate and certain intermediates of the TCA cycle in the absence of nitrite oxidation, supportive data involving enzyme assays with cell-free extracts are needed to determine if glyoxylate can be metabolized by an energy generating and biosynthetic pathway. Since our growth studies indicate that growth is not supported by acetate it appears that either the combination of an energy yielding and biosynthetic pathway is nonexistant or the energy generated from such a pathway can not be coupled to other necessary biosynthetic processes.

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