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Nitrate Reduction and the Growth of *Veillonella alcalescens*

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Veillonella alcalescens, a strict anaerobe, was found to possess a nitrate reductase system which has characteristics of both assimilatory and respiratory nitrate reduction. The nitrate reductase has been identified tentatively as a particulate enzyme which utilizes a variety of electron donors for the reduction of nitrate. By use of ^{15}N-labeled nitrate, it was shown that under appropriate conditions nitrate nitrogen is incorporated into cell material. *V. alcalescens* grown on pyruvate and nitrate has a greater growth rate than cells grown on pyruvate alone. Growth can occur in a medium with hydrogen and nitrate as the sole energy source. Ammonium chloride decreases the rate of nitrate reduction but does not completely inhibit reduction or incorporation. The results suggest that nitrate assimilation and respiration are not as distinct as in some other organisms.

The only reported energy mechanism occurring in *Veillonella* is the phosphoroclastic system described by McCormick, Ordal, and Whiteley (9). Valentine and Wolfe (12) showed that ferredoxin was either essential or greatly stimulated pyruvate oxidation in a variety of organisms, including *Veillonella*. In each organism the ferredoxin was linked to a hydrogenase leading to the formation of molecular hydrogen. Whiteley and Woolfolk (15) showed that the hydrogenase was fully reversible and hydrogen oxidation could be coupled to the reduction of a variety of compounds of the metallic and transition elements and organic compounds. They also demonstrated that both nitrate and hydroxylamine were reduced to the level of ammonia by molecular hydrogen with cell extracts of *Veillonella*. In these experiments added ferredoxin was not essential for the reduction of nitrate, but the reduction was greatly stimulated by methyl viologen.

Verhoeven (13), Nason (10), and others classified nitrate reduction in bacteria into two types based on physiological function: (i) nitrate assimilation, in which nitrate is reduced to the level of ammonia and incorporated into cell material, and (ii) nitrate respiration, in which energy is produced as a result of the transport of electrons to nitrate.

Differentiating between the two types of nitrate reduction systems, except on the basis of function, has proven to be difficult. Garrett and Nason (1) showed that both assimilatory and respiratory reductases may be cytochrome linked. They suggested that the location of the reductase in the cell is more indicative of the type of nitrate reduction, the dissimilatory enzymes usually being found in a particulate state. This latter generalization must be considered with reservation, however, for a soluble respiratory nitrate reductase has been reported in *Spirillum itersonii* (2). It appears, however, that all assimilatory nitrate reductases are inhibited or repressed by ammonium salts, whereas respiratory reductases are not inhibited (4, 5, 11, 16).

MATERIALS AND METHODS

Veillonella alcalescens C1 was used in this study. The strain was isolated from sheep rumen and has been maintained in this laboratory for a number of years. Stocks of the organism were maintained as stab cultures in a medium of 1.0% sodium lactate (60% syrup, Fisher Chemical), 1.0% yeast extract (Difco), 1.0% tryptone (Difco), and 1.5% agar (Difco). For measurement of growth rate and nitrate utilization, cells were grown on a basal medium containing 0.1% yeast extract, 0.1% tryptone, and 1.0% casein hydrolysate (Nutritional Biochemicals, Inc.) and, when added, sodium pyruvate, 100 mM, and potassium nitrate, 1 mM to 50 mM. The media were made to volume with 50 mM potassium phosphate buffer (pH 7.0) and autoclaved separately from the casein hydrolysate, which was added after sterilization.

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Larger amounts of cells for preparation of cell-free extracts were grown in 5- or 10-liter volumes of the liquid stock culture medium. Cultures in early exponential growth phase were chilled with crushed ice, and the cells were harvested by centrifugation at 20,000 × *g* for 20 min at 5 C. The cell pellet was suspended in 5 to 10 ml of potassium phosphate buffer (pH 7.0). This suspension was placed in an "X-press" (Biochemical Processes, Inc., Islip, N.Y.), frozen, subjected to a pressure of 25,000 lb/in² at −25 C, thawed, and treated with a few crystals of deoxyribonuclease and ribonuclease to decrease the viscosity of the preparation. Whole cells and debris were removed by centrifugation at 10,000 × *g* for 20 min. The extracts either were used as such or were fractionated in a preparative ultracentrifuge. The crude extracts usually contained 40 to 60 mg of protein per ml, and when fractionated were diluted 1:10 with phosphate buffer before ultracentrifugation at 140,000 × *g* for 3 to 6 h. The pellet fraction was designated P-144, the supernatant fraction was designated S-144, and the crude extract was designated S-10. All extracts were stored at −20 C under nitrogen or helium. Protein was determined either by the biuret method (3) or by the method of Lowry (8) with crystalline bovine serum albumin as the standard. Nitrite was determined by the method of John and Whatley (6). A sample of the growth medium or deproteinized extract was made to a volume of 1.0 ml with water. To this volume was added 5 ml of 1% sulfanilamide in 1 N HCl and 4 ml of 0.1% *N*-(*1*-napthyl)-ethylene diamine-dihydrochloride. The absorbancy was measured at 540 nm after 20 min. Reagent grade sodium nitrite was used as the standard.

Routine assay of the nitrate reductase was by the method of Gauthier et al. (2) with only slight modification. Sufficient extract was added to each tube to yield approximately 0.5 μmol of nitrite per min. The assay mixture contained in micromoles: potassium nitrate, 20; benzyl viologen, 0.1; sodium dithionite, 4.6, in solution with sodium bicarbonate, 4.6; and potassium phosphate buffer (pH 7.0), 80; in a total volume of 1.0 ml. Nitrate was not reduced by dithionite alone, and the assay mixtures remained anaerobic if placed in 10 mm by 100 mm tubes. Nitrate reduction with either reduced flavine-adenine dinucleotide (FAD) or flavine mononucleotide (FMN) was performed as above except that 0.1 μmol of flavine was substituted for benzyl viologen. Nitrate reduction with reduced pyridine nucleotides was determined either by following the oxidation of nicotinamide adenine dinucleotide, reduced form (NADH), or nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), at 340 nm in a Thunberg cuvette or by assaying for the production of nitrite in Thunberg tubes containing substrate levels of the reduced pyridine nucleotides. The spectrophotometric assay contained in μmoles: tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 100; potassium nitrate, 2; NADH or NADPH, 0.4; and 0.1 to 0.5 mg of extract protein in a total volume of 1.0 ml. Assays in Thunberg tubes contained in micromoles: tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 100; potassium nitrate, 20; NADH or NADPH, 5; and 0.5 to 1.0 mg of extract protein in a total volume of 3.0 ml. The NADH or NADPH was placed in the side arm of the tube or cuvette which was then evacuated three times and refilled with nitrogen. Nitrate reduction with molecular hydrogen was determined manometrically. The assay mixture contained in micromoles: potassium nitrate, 20; FAD or FMN, 0.1; or benzyl viologen, 0.5; MgCl, 20; potassium phosphate buffer (pH 7.0), 150; and 5 to 10 mg of protein in a total volume of 3.0 ml with 0.2 ml of 20% KOH in the center well. The flasks were gassed for 10 to 15 min or until the flavine or benzyl viologen was reduced. The reaction was started with the addition of nitrate and terminated with the addition of 0.1 ml of a saturated zinc acetate solution. After adding 4.9 ml of 95% ethanol, the precipitated protein was removed by centrifugation at 3,000 × *g*, and the supernatant fluid was assayed for nitrite. All assays were performed at 37 C. Unless otherwise noted, specific activity is in terms of nanomoles of nitrite produced per minute per milligram of protein.

The growth rates in various media were determined by periodically sampling a 50-ml batch culture and measuring the optical density at 650 nm. Nitrite accumulation in the medium was assayed after an appropriate dilution with water. The cultures were maintained at 37 C ± 0.1 in a circulating water bath and were continuously stirred with a small magnetic stirrer. The cultures were grown under an atmosphere of either nitrogen or hydrogen by continuous sparging. Residual oxygen in the nitrogen or hydrogen was removed by bubbling the gas through 5% pyrogallol in 5 N NaOH and finally through water to remove entrained alkali. To determine precisely the growth rates in the various media, the cultures were serially diluted into the same medium to maintain the cells in exponential growth phase for three to six generations. To determine the course of nitrate reduction and incorporation, cultures were allowed to proceed through the usual batch culture growth curve without dilution. After determining the final optical density and nitrite concentration, the cells were harvested by centrifugation at 10,000 × *g* and washed three times with 0.1 M potassium phosphate buffer (pH 7.0). The final cell pellet was dried under vacuum over phosphorous pentoxide.

The incorporation of ^{15}N-labeled nitrate into cell material was determined on the dried cells by the Kjeldahl method and mass spectrometry.

RESULTS

Growth studies. Pritchard (Ph.D. thesis, Cornell Univ., Ithaca, N.Y., 1969) reported an increased molar growth yield for *V. alcalescens* in a complex medium with nitrate and pyruvate as compared to pyruvate alone, but only if the nitrate concentration was 10 mM or less. Over the range of 1 to 50 mM nitrate the molar growth yield varied from 20 to 4 g dry weight of cells per mole of nitrate, suggesting that high nitrate concentrations, or nitrite concentrations, inhibited growth.

Figure 1 shows the results of an experiment in

which nitrate utilization was determined with cells growing on 0.1 M pyruvate with various concentrations of nitrate. At nitrate concentrations of 10 mM or less, there was a transitory appearance of nitrite in the growth medium. At higher nitrate concentrations, nitrite continued to accumulate in the medium as the cells entered the stationary phase of growth, and the nitrite was never completely utilized. At each nitrate concentration the growth rate of the organism was identical and substantially higher than the growth rate in the same medium without nitrate. In Table 1 are shown the growth rates of *V. alcalescens* grown on pyruvate with and without nitrate. The growth rate on pyruvate was identical to that reported by Michaud (Ph.D. thesis, Cornell Univ., Ithaca, N.Y., 1968) and by Pritchard (Ph.D. thesis, Cornell Univ., Ithaca, N.Y., 1969), but in the presence of nitrate the specific growth rate increased to an average value of 0.77/h. Since it has been shown in cell-free extracts of this organism that the hydrogenase reaction is rate limiting for the phosphoroclastic system, nitrate reduction might provide a more efficient electron sink or may serve as an alternative energy-producing system. If the latter was true, however, then *V. alcalescens* could have been grown on molecular hydrogen and nitrate alone. In Fig. 2 are shown the results when a culture was grown in a complex medium with hydrogen and nitrate as the only energy source. Under these conditions nitrate was reduced only to nitrite. If the cultures were sparged with hydrogen for a prolonged period of time, there was some nitrite reduction. There was no growth in the absence of nitrate and only slight growth when nitrogen was substituted for hydrogen. As shown in Table 1, the specific growth rate on hydrogen and nitrate was lower than on either pyruvate or nitrate and pyruvate.

Cells grown on hydrogen and nitrate required 1% casein amino acids for growth, whereas cells grown on nitrate and pyruvate did not require the added amino acids, although the total growth was enhanced. In other experiments (not shown) it was found that, when cells were grown on a very low concentration of nitrate (1 mM),

TABLE 1. *Growth constants of V. alcalescens C-1 on various substrates*

Substrate	Average specific growth rate	No. of experiments
Pyruvate	0.52	7
Pyruvate + nitrate	0.77	7
Hydrogen + nitrate	0.38	5

[a] Growth rates determined as described in Materials and Methods in media with 0.1 M sodium pyruvate, 25 mM KNO_3 and 0.1 M sodium pyruvate, or 50 mM KNO_3 and molecular hydrogen. Specific growth rate is ln 2/doubling time.

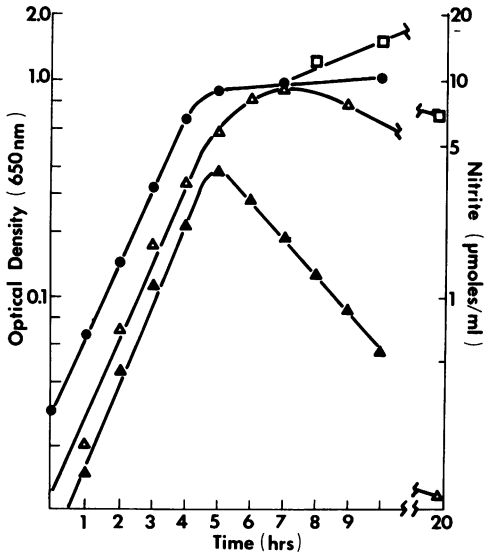

FIG. 1. *V. alcalescens growth and nitrite production in media containing different amounts of nitrate. Growth as optical density (650 nm) for each medium (●) and nitrite production in media with 20 mM KNO_3 (□), 10 mM KNO_3 (△), and 5 mM KNO_3 (▲).*

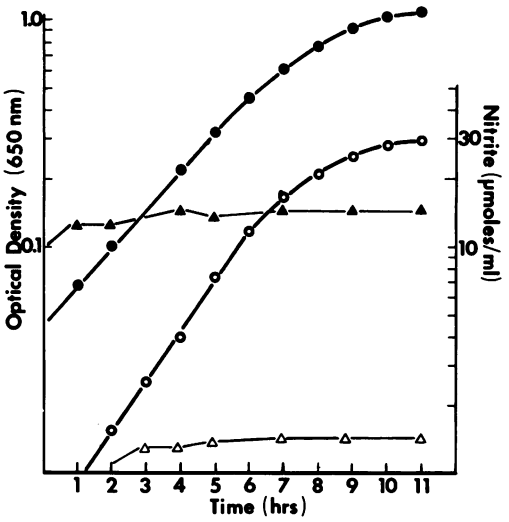

FIG. 2. *Growth of V. alcalescens on hydrogen and 50 mM nitrate. Growth as optical density (650 nm) on hydrogen and nitrate (●); nitrogen and nitrate (○). Nitrite production on hydrogen and nitrate (▲); nitrogen and nitrate (△).*

the growth stimulation ceased when the nitrate supply was exhausted, even though nitrite reduction ensued immediately.

Incorporation studies. Since Whiteley and Woolfolk (15) had shown that extracts of *V. alcalescens* could reduce nitrate to hydroxylamine and ammonia with hydrogen, it was of interest to determine the fate of nitrate nitrogen in cultures as described above. In Fig. 3 are shown the results of an experiment in which *V. alcalescens* was grown in media containing pyruvate and ^{15}N-labeled nitrate and in the presence or absence of 40 mM ammonium chloride. When grown in a medium with 5 mM nitrate and 40 mM ammonium chloride, the specific growth rate of the culture decreased to 0.58/h, or slightly more than the specific growth rate of a culture grown on pyruvate alone. Under these conditions, nitrite accumulated until the cells entered the stationary phase of growth, after which nitrite production ceased. Approximately 32% of the available nitrate was reduced to nitrite; there was a protracted inactivity on nitrite, but eventually nearly all of the nitrite (27%) was reduced and incorporated into cell material. When cells were grown in a medium with 5 mM potassium nitrate and no ammonium chloride, the nitrate was rapidly reduced to nitrite. The accumulated nitrite was utilized directly with 68% on the ^{15}N-labeled nitrate nitrogen incorporated into cell material (Table 2). It should be noted that the incorporation experiments were done with cells grown in a complex medium containing casein amino acids, yeast extract, and tryptone. Since the total nitrogen content of these compounds is variable, ranging from 12 to 14% total nitrogen, the incorporation figures should not be considered absolute.

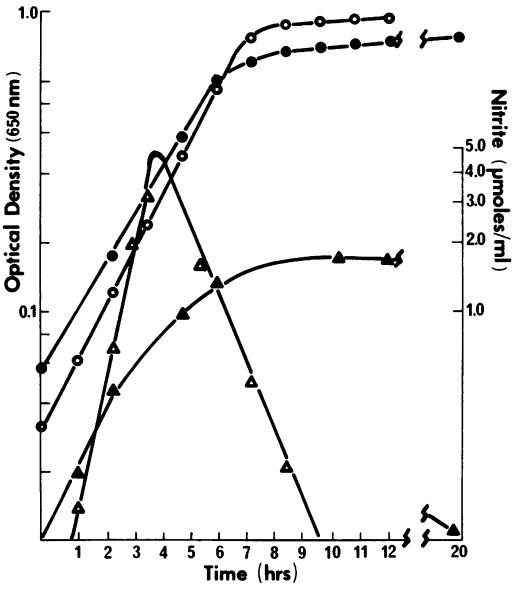

FIG. 3. *V. alcalescens growth and nitrite production in a medium containing 0.1 M pyruvate, 5 mM KNO_3, and with or without 40 mM NH_4Cl. Growth as optical density (650 nm) for the medium without NH_4Cl (○) and with NH_4Cl (●). Nitrite production in the medium without NH_4Cl (△) and with NH_4Cl (▲).*

When *V. alcalescens* was grown on hydrogen and nitrate in the presence of 40 mM ammonium chloride, the specific growth rate of the culture was slightly lower (0.33/h) than in the absence of ammonia.

Nitrate reductase. The nitrate reductase from *V. alcalescens* was characterized to determine any similarities to assimilatory and respiratory nitrate reductases in other organisms.

In Table 3 are shown the results of an experiment which compares the nitrate reductase activities of extracts from cells grown in the presence and absence of nitrate. With both reduced benzyl viologen and reduced FAD there was a marked increase in the specific activity of the nitrate reductase from cells grown in the

TABLE 2. *Incorporation of ^{15}N-labeled nitrate*[a]

Growth conditions	Atom (%) of ^{15}N in medium	Atom (%) of ^{15}N in cells	Incorporation (%)
5 mM Nitrate	1.86	1.26	68
5 mM Nitrate + 40 mM NH_4^+	1.34	0.367	27

[a] Effect of growth conditions on the incorporation of ^{15}N-labeled nitrate into cell material. Media contained pyruvate, tryptone, yeast extract, casein amino acids, and buffer as described in Materials and Methods.

TABLE 3. *Effect of nitrate on specific activity of nitrate reductase*

Medium[a]	Electron donor[b]	Specific activity nmol/min/mg
+ Nitrate	BV^+	669
− Nitrate	BV^+	10
+ Nitrate	$FADH_2$	37
− Nitrate	$FADH_2$	1

[a] Cells were grown in a complex medium containing 1.0% yeast extract, 1.0% tryptone, 0.1 M sodium pyruvate, and plus or minus 50 mM KNO_3.

[b] Electron donors were reduced with dithionite: benzyl viologen (BV^+) and flavine adenine dinucleotide ($FADH_2$).

presence of nitrate. The specific activity of the nitrate reductase from cells grown in the absence of nitrate was approximately the same as reported by Whiteley and Woolfolk (15). FMN substituted for FAD without a noticeable difference in the specific activity. It should be noted that there was activity present in cells grown in the absence of nitrate. This indicates that the reductase either is, in part, constitutive and stimulated in the presence of nitrate, or that there is a nonspecific reductase present which reduces nitrate inefficiently compared to an inducible nitrate reductase.

To make a presumptive determination as to the location of the nitrate reductase in the cell, a cell-free extract was separated into soluble and particulate fractions in a preparative ultracentrifuge. The specific activities of the three fractions are compared in Table 4. As shown, more than 80% of the nitrate reductase activity appeared in the particulate fraction. When the preparation was centrifuged for longer periods of time, more of the soluble nitrate reductase appeared in the particulate fraction (not shown). The nitrate reductase is stable when stored under nitrogen or helium at −20 C for several weeks. The activity of the enzyme diminishes rapidly when the preparation is frozen and thawed.

A comparison of the nitrate reductase activities with various electron donors is shown in Table 5. The activities reported for molecular hydrogen, with or without added factors, were somewhat variable because of the lability of the hydrogenase. A similar hydrogenase lability was reported in *V. alcalescens* by Whiteley and Ordal (14). The activities shown in Table 5 are for enzyme preparations of equal activity. The benzyl viologen coupled hydrogenase-nitrate reductase activity was lower than the dithionite-reduced benzyl viologen activity. Also, the activity with dithionite-reduced FAD was higher than with either of the activities with flavine coupled hydrogenase and nitrate reductase. These findings suggest that the limiting step is the hydrogenase, when molecular hydrogen is the electron donor. If the cell preparation was sufficiently active and initially contained a high concentration of protein (50–60 mg/ml), no extra factors were required to couple the hydrogenase and the nitrate reductase.

TABLE 4. *Distribution of nitrate reductase*

Fraction[a]	Specific activity (nmol/min/mg)	Total units[b] μmol/min)	(%)[c]
S-10	513	38.5	100
S-144	115	4.6	12
P-144	918	32.1	84

[a] Fractions are as described in Materials and Methods.

[b] Total units are the specific activities times total protein.

[c] Distribution of nitrate reductase activity in 144 times fractions as a percentage of the S-10 fraction.

TABLE 5. *Reduction of nitrate with various electron donors*

Donor system	Specific activity (nmol/min/mg)
H_2[a]	14
H_2 (FAD)[b]	10
H_2 (FMN)[b]	10
H_2 (BV)[b]	80
$FADH_2$[c]	37
BV^+[c]	669
	OD/min/mg[d]
NADH[d]	<0.01
NADPH[d]	<0.01

[a] Oxidation of H_2 with nitrate without added factors.

[b] Oxidation of H_2 with nitrate with FAD, 0.1 mM; FMN, 0.1 mM; or benzyl viologen (BV^+), 1 mM.

[c] Reduction of nitrate with dithionite-reduced FAD or BV.

[d] Nitrate reduction assayed as the change in optical density (OD) at 340 nm, activity not detectable but less than the indicated value. All assays were done with the S-10 fraction of a cell-free extract.

Neither NADH nor NADPH donated electrons to the nitrate reductase. Also, neither FAD nor FMN had an effect on the inactivity with the reduced pyridine nucleotides, and there was no observable NADH or NADPH oxidase activity with any of the preparations tested.

Since the hydrogenase is a soluble enzyme and the flavine-linked activity was often lost upon fractionation, all of the nitrate reductase experiments were performed with the S-10 fraction of cell extracts. For all of the enzyme studies it was assumed that nitrate was reduced only to nitrite. Contrary to the report of Whiteley and Woolfolk (15), there was no indication of nitrate reduction to the level of ammonia with cell-free extracts of this strain of *V. alcalescens*. As shown in Table 6, the reduction of nitrate with molecular hydrogen with benzyl viologen gave stoichiometric evidence that nitrate was not reduced beyond nitrite in our test system.

DISCUSSION

We showed that the rate of growth of *V. alcalescens* on pyruvate is stimulated by ni-

TABLE 6. *Stoichiometry of nitrate reduction with hydrogen*[a]

Flask no.	μmol of NO_2^-	μmol of H_2	H_2/NO_2
1	11.4	11.36	0.99
2	10.8	7.51	0.70
3	10.5	9.80	0.93
4	10.5	9.04	0.86

[a] H_2 uptake determined manometrically and nitrite determined as described in Materials and Methods.

trate. At appropriate concentrations, one observes the reduction of nitrate to nitrite and the subsequent incorporation of nitrate nitrogen into cell material. The molar growth yield data of Pritchard (Ph.D. thesis, Cornell Univ., Ithaca, N.Y., 1968) and the evidence presented here suggest an alternative energy mechanism to the known phosphoroclastic system. Although nitrate also may provide a more efficient mechanism for disposing of the electrons from the phosphoroclastic reaction, this could not account for the increased molar growth yields on nitrate and the lithotrophic growth on hydrogen and nitrate.

Since cells only reduced nitrate to nitrite when grown on hydrogen and nitrate, an alternative source of electrons may be required for the reduction of nitrite. The requirement for amino acids when growing on hydrogen and nitrate may reflect the little understood processes of hexose and pentose biosynthesis in this organism. Michaud (Ph.D. thesis, Cornell Univ., Ithaca, N.Y., 1968) has found, for example, that a small amount of radioactively labeled lactate or pyruvate was incorporated into cell material and proposed a scheme of pentose biosynthesis from glycolytic and tricarboxylic acid cycle intermediates. When grown on hydrogen and nitrate, *V. alcalescens* may require larger and more diverse pools of amino acids for biosynthetic functions. It is not clear whether there is a biochemical or physiological separation of assimilatory and respiratory nitrate reduction in *V. alcalescens*. Evidently ammonia inhibits the reduction of nitrate to nitrite to a limited extent, manifested as a change in the rate of nitrate reduction and the degree of ^{15}N incorporation. In addition, in many bacteria nitrate reduction is repressed if there are alternative nitrogen sources present, such as casein amino acids. In all of the incorporation studies reported here, casein amino acids were present, yet nitrate reduction and incorporation was demonstrated.

The presumptive data on the nitrate reductase indicates that it cannot utilize reduced pyridine nucleotides. It was not shown whether this nitrate reductase was cytochrome linked, but *Veillonella* has been reported not to contain cytochromes (7). Flavines, FAD and FMN, can donate electrons as well as benzyl viologen, when reduced either with hydrogen or dithionite. Other unpublished data shows that the reductase is inhibited by cyanide. These findings suggest that the nitrate reductase may have some similarities to the nitrate reductases in other organisms. Cell-free assays of the nitrate-linked phosphorylation mechanism and further characterization of the nitrate reductase are presently being undertaken.

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