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Energy metabolism and biosynthesis of *Vibrio succinogenes* growing with nitrate or nitrite as terminal electron acceptor

Martin Bokranz, Joachim Katz, Imke Schröder, Anthony M. Roberton, and Achim Kröger

Fachbereich Biologie, Mikrobiologie, Philipps-Universität, D-3550 Marburg, Federal Republic of Germany

Abstract. 1. Growth of *Vibrio succinogenes* with nitrate as terminal electron acceptor was found to be a function of the following two catabolic reactions:

$$HCO_2^- + NO_3^- + H^+ \rightarrow CO_2 + NO_2^- + H_2O \quad (a)$$

$$3\,HCO_2^- + NO_2^- + 5\,H^+ \rightarrow 3\,CO_2 + NH_4^+ + 2\,H_2O. \quad (b)$$

The latter reaction (b) was responsible for growth with nitrite.

2. Either succinate or fumarate could serve as sole carbon source during growth with nitrate or nitrite. Biosynthesis from succinate proceeded *via* fumarate. The ATP requirement for cell synthesis from succinate was equal to that calculated earlier for growth with fumarate as carbon source and electron acceptor (Brounder et al. 1982).

3. The cell yield at infinite dilution rate (Y^{max}) as obtained with chemostat cultures was 8.5 g dry cells/mol formate with either nitrate or nitrite as acceptor. This value amounts to 60 % of that measured earlier with fumarate as acceptor (Mell et al. 1982).

4. Membrane vesicles prepared from *V. succinogenes* catalyzed electron transport from H_2 to nitrite. The reaction was dependent on the menaquinone present in the membrane.

5. Electron transport with H_2 and nitrite was coupled to the phosphorylation of ADP. The P/H_2 ratio with nitrite was 40 % of that measured with fumarate as acceptor using the same preparation. The phosphorylation but not the electron transport was abolished by an uncoupling agent.

Key words: Phosphorylative nitrite reduction – Nitrate reduction – *Vibrio succinogenes*

Introduction

Vibrio succinogenes is known to gain ATP from electron transport phosphorylation with either fumarate or nitrate as terminal acceptor (Wolin et al. 1961). The stoichiometric ATP gain with fumarate was measured to be nearly 1 ATP/2 electrons (Kröger and Winkler 1981; Mell et al. 1982). In this communication it is shown that *V. succinogenes* performs electron transport phoshorylation also with nitrite as terminal acceptor. The ATP gain of this reaction is compared to that with nitrate or fumarate as acceptor. For this purpose cell yields from growth on a minimal medium as well as phosphorylation yields with a membrane preparation were measured.

V. succinogenes was proposed to be renamed as *Wolinella succinogenes* (Tanner et al. 1981). The strain used here was obtained from Wolin (Wolin et al. 1961) and is identical with ATCC 29543.

Offprint requests to: A. Kröger

Abbreviations: TRIS, tris (hydroxymethyl)aminomethane; MOPS, 3-(N-morpholino)propanesulfonate; MK, menaquinone; FCCP, carbonyl-cyanide-4-trifluoromethoxyphenylhydrazone; EDTA, ethylene diamine tetraacetate

Materials and methods

Growth media

The growth medium contained 50 mM TRIS, 1 mM K_2HPO_4, 5 mM K_2SO_4, 1 mM $MgCl_2$ and 1 ml/l of the trace element solution described earlier (Bronder et al. 1982). 40 mM Na-formate, 10 mM $NaNO_3$, 10 mM succinate (disodium salt), 0.68 mM glutamate and 0.57 mM cysteine were present, unless indicated otherwise. Alanine (1.1 mM), tryptophan (0.49 mM) or yeast extract (1 g/l) were present when indicated. $MgCl_2$, the amino acids and the yeast extract were sterilized separately. H_2S (1 %) was supplied in a mixture with N_2, which was passed through the culture at 3 l/h. The optimum growth temperature was 37° C.

Growth of V. succinogenes

In batch culture experiments the bacteria were grown at 37° C in stoppered 0.5 l-flasks which were equipped with tubes for passing N_2 through the culture (250 ml). The medium was brought to pH 7.5, and inoculated with 10 % of a preculture (Bronder et al. 1982). Then N_2 was passed through the culture for 15 min during stirring. Continuous culture was carried out as described earlier (Mell et al. 1982).

Analytical procedures

Cell density (Bronder et al. 1982), nitrate (Hartley and Asai 1963), nitrite (Rider and Mellon 1946), formate (using NAD and formate dehydrogenase) and ammonium (Bergmeyer 1974) were determined as described.

Nitrite reduction by H_2 was measured using a Clark-type hydrogen electrode. The membrane preparation was incubated in 10 mM TRIS pH 7.9 saturated with H_2. The reaction was started by adding 10 mM $NaNO_2$.

Nitrite reduction by reduced benzyl viologen was measured photometrically at 546 nm. The membrane preparation was suspended in anaerobic phosphate buffer (50 mM) pH 6.6 containing 0.3 mM benzyl viologen. After adding 0.5 mM

$Na_2S_2O_4$ the reaction was started by the addition of 10 mM $NaNO_2$.

Phosphorylation was measured with membrane vesicles prepared from fumarate-grown *V. succinogenes* as described (Kröger and Winkler 1981). The vesicles (0.6 g protein/l) were shaken under an atmosphere of H_2 in a buffer containing 0.25 M mannitol, 10 mM MOPS, 25 mM $MgCl_2$, 25 mM glucose, 10 mM [^{32}P]phosphate, 1 mM ADP, 2.5 g/l purified bovine serum albumin and 0.25 μkat/ml dialyzed hexokinase at pH 7.5 and 37°C. Electron transport was started by the addition of either nitrite or fumarate. After complete reduction of the acceptor, $HClO_4$ (0.3 M) was added and the extract analyzed for organic phosphate.

Results

Biosynthesis

When grown with fumarate as terminal electron acceptor, *Vibrio succinogenes* was found to use fumarate as the sole carbon source (Bronder et al. 1982). With nitrate as the terminal acceptor and formate as the donor substrate, *V. succinogenes* grew on either succinate or fumarate as the sole carbon source (Table 1). The bacteria did not grow, when fumarate or succinate was replaced by a mixture of glutamate, alanine, cysteine and tryptophan (not shown), in agreement with Niederman and Wolin (1972). Cysteine or H_2S was required for growth with nitrate, and could not be replaced by sulfate. In contrast sulfate was used as a source of sulfur with formate and fumarate as catabolic substrates (Bronder et al. 1982). The growth rates and the cell yields were slightly increased when glutamate or yeast extract was present in addition to succinate or fumarate.

With [2,3-^{14}C] succinate as the sole carbon source the growing bacteria were found to incorporate equivalent amounts of succinate (14 mmol/g dry cells) and radioactivity from the culture fluid (not shown). This indicates that succinate is used merely for cell synthesis and is not converted to other products (except for CO_2). The main pathways of biosynthesis were traced by comparing the specific radioactivity of the alanine, aspartate and glutamate residues of the protein of the bacteria to that of the succinate (Table 2). The specific radioactivities were measured after hydrolysis of the bacterial protein and isolation of these amino acids. Division of the specific radioactivity values by that of the succinate gave the contents of labeled carbon relative to that of succinate. These radioactivity ratios indicate that each alanine and aspartate residue was formed from 1 molecule of succinate, while glutamate was synthesized from 2 molecules of succinate. Therefore, it is concluded that biosynthesis from succinate proceeds via fumarate. Glutamate is formed via citrate and not via reductive carboxylation of succinyl-CoA. Similar results were earlier obtained with [2,3-^{14}C] fumarate as carbon source and terminal acceptor.

During growth with glutamate in addition to succinate in the medium, the glutamate carbon was predominantly incorporated into the amino acids of the glutamate family (glutamate, glutamine, arginine and proline) (not shown). A smaller part of the glutamate was apparently converted to succinate which was excreted into the medium. This suggests that glutamate oxidation to succinate is used as a biosynthetic hydrogen source (Bronder et al. 1982) also during growth with nitrate. The carbon of the cysteine which was offered as additional biosynthetic substrate, was exclusively incorporated into the alanine residues of the bacterial protein (not shown).

Table 1. Doubling times and growth yields of *V. succinogenes* grown with formate and nitrate

Additions to growth medium	Doubling time (h)	*Y* (g dry cells/mol formate)
Succinate + H_2S	1.8	4.7
Succinate + cysteine	1.6	4.6
Succinate + cysteine + glutamate	1.4	5.2
Succinate + yeast extract	1.3	5.6
Fumarate + cysteine	1.6	4.9
Fumarate + cysteine + glutamate	1.4	5.6

Table 2. Ratios of specific radioactivity of glutamate, aspartate and alanine residues from *V. succinogenes* protein, compared to that of [2,3-^{14}C] succinate. The bacteria were grown in batch culture as described in Materials and Methods. However, the concentration of succinate was 4 mM (138 Bq/μmol) and H_2S was passed through the culture, glutamate and cysteine were absent. The specific radioactivity of the amino acids was determined after isolation from the protein according to Fuchs et al. (1978)

Amino acid *analyzed*	Radioactivity *ratio*
Glutamate	1.73
Aspartate	1.00
Alanine	0.99

Energy metabolism

Stoichiometry of growth reactions

The catabolism during growth with nitrate as acceptor is described by reaction (a) and (b). This is shown by the experiment of Fig. 1, where cell density and the concentrations of formate, nitrate, nitrite and ammonium were measured in a growing batch culture. The concentration of nitrite increased 3 times faster than that of NH_4^+ as long as nitrate was present in the medium (Fig. 1 A).

$$HCO_2^- + NO_3^- + H^+ \rightarrow CO_2 + NO_2^- + H_2O \quad \text{(a)}$$

$$3\,HCO_2^- + NO_2^- + 5\,H^+ \rightarrow 3\,CO_2 + NH_4^+ + 2\,H_2O \quad \text{(b)}$$

The slope of line I in Fig. 1 B (53 mmol N/mol formate) represents the theoretical amount of nitrogen required for cell formation. This number was calculated from the cell yield (5.3 g dry cells/mol formate) on the basis that 10 mmol N/g dry cells is needed. The experimental points (triangles in Fig. 1 B) which were obtained as the differences between the amounts of nitrate consumed and those of nitrite and NH_4^+ produced, are fairly consistent with the theoretical line I. It is concluded that the amount of nitrate consumed is equal to the sum of the amounts of nitrite and of free and cellular NH_4^+ produced.

The slope of line II (Fig. 1 B) indicates that 0.86 mol[2H]/mol formate is expected to be recovered in the nitrite and the free NH_4^+. The residual 0.14 mol[2H]/mol formate represent the amount of formate used for biosynthetic NH_4^+ formation. This number is obtained from the difference between the total

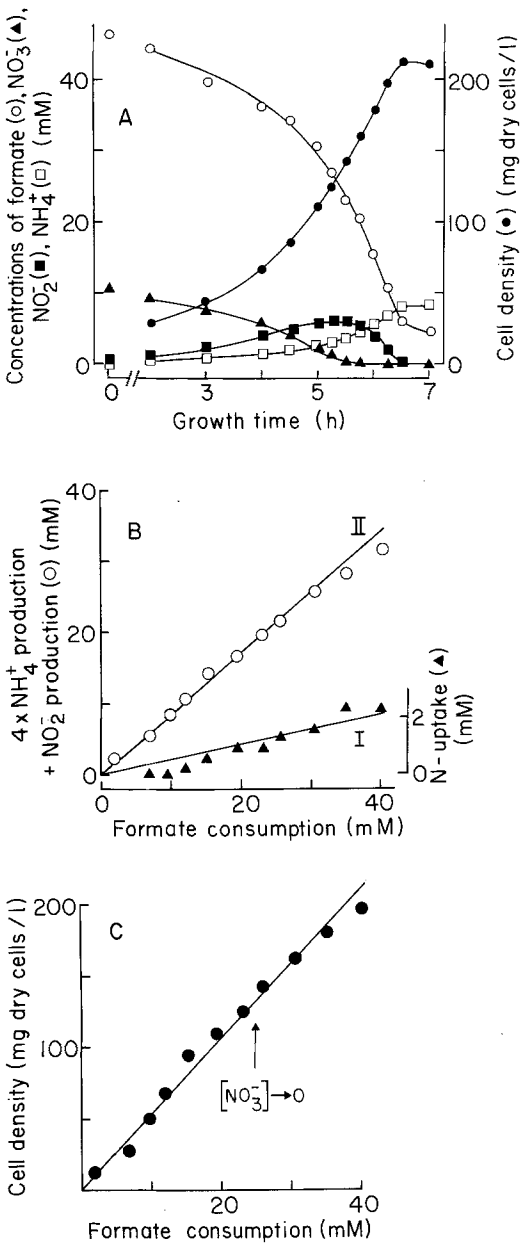

Fig. 1A–C. Batch culture of *V. succinogenes* growing on formate and nitrate. Glutamate was absent. **A** Cell density and the concentration of substrates and products as a function of growth time. **B** Nitrogen uptake and reducing equivalents recovered in nitrite and NH_4^+ plotted against formate consumption. The theoretical lines *I* and *II* were obtained as described in the text. **c** Cell density as a function of formate consumption

amount of hydrogen required for biosynthetic NH_4^+ formation from nitrate (40 mmol[2H]/g dry cells) and that supplied by succinate oxidation to fumarate (14 mmol[2H]/g dry cells), on multiplication with the cell yield. The dots calculated from the experimentally determined amounts of nitrite and free NH_4^+, closely follow the theoretical line II. It is concluded that the reducing equivalents of the formate consumed were recovered in the nitrite, the free and the cellular NH_4^+.

In summary, the data of Fig. 1B show that nitrite and NH_4^+ are the only products of nitrate reduction.

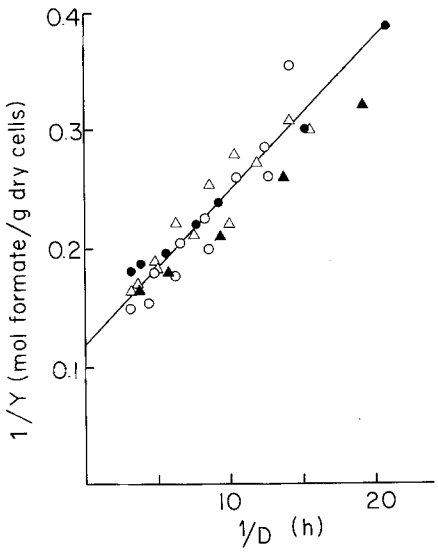

Fig. 2. Double reciprocal plots of growth yields (Y) against dilution rates (D) from continuous cultures of *V. succinogenes*. Concentrations in the media: 50 mM formate and 10 mM nitrate (○), 45 mM formate and 15 mM nitrate (●), 41 mM formate and 10 mM nitrite (△), 22.6 mM formate and 10.4 mM nitrite (▲). The cell densities (for calculating Y) were measured using dry weight determination

Furthermore, the data confirm that succinate as the carbon source is oxidized by nitrate. Finally, it is shown here again that cell synthesis from fumarate and NH_4^+ neither requires nor liberates significant amounts of reducing equivalents (Bronder et al. 1982).

From Fig. 1A it can be calculated that about half the formate is oxidized according to reaction (a), as long as nitrate is present in the culture. After the exhaustion of nitrate, growth was sustained by reaction (b) alone. Cell formation was a linear function of the amount of formate consumed, and the cell yield (5.3 g dry cells/mol formate) was the same with and without nitrate present (Fig. 1C). This indicates that *V. succinogenes* performs electron transport phosphorylation with nitrate as well as with nitrite as acceptor. In a similar experiment (not shown) with nitrate (50 mM) in excess over formate (25 mM), 80 % of the formate was oxidized according to reaction (a), and nearly the same cell yield (5.5 g dry cells/mol formate) was measured as in the experiment of Fig. 1. Both experiments suggest equal ATP gains of reaction (a) and (b).

Growth in continuous culture

V. succinogenes was grown in continuous culture with either nitrate (dots) or nitrite (triangles) as acceptor (Fig. 2). Succinate, glutamate and cysteine were present as biosynthetic substrates under all conditions. Cultures were kept at pH 7.8 by means of a pH-stat.

When the medium contained 50 mM formate and 10 mM nitrate (○), neither nitrate nor nitrite could be detected in the culture fluid. This indicates that growth was limited by the concentrations of the acceptors. With 45 mM formate and 15 mM nitrate in the medium (●) nitrate and formate were absent from the culture fluid and the concentration of nitrite was 5 mM. Hence growth was limited by the concentration of formate and 1/3 of the formate was oxidized according to reaction (a) under these conditions.

V. succinogenes grew with nitrite in continuous culture both with nitrite or formate at limiting concentration. Growth was limited by nitrite with 41 mM formate and 10 mM nitrite in the medium (△), since nitrite was not detected in the culture fluid. Formate was growth limiting with 22.6 mM formate and 10.4 mM nitrite in the medium (▲).

The cell yields (Y) measured in the steady states established at each dilution rate (D) were related to D according to Eq. (1). Double reciprocal plots of the values of Y measured under the various growth conditions at the corresponding dilution rates fit approximately to a common straight line (Fig. 2). This line is characterized by $Y^{max} = 8.5$ g dry cells/mol formate and by a maintenance coefficient $m = 13$ mmol formate/g dry cells per h. When succinate was replaced by fumarate as the carbon source in the medium, nearly the same values of Y^{max} and m were obtained (not shown). Succinate, but no fumarate, was found in the culture fluid under these conditions.

$$1/Y^{max} = 1/Y + m/D \quad (1)$$

Menaquinone-dependence of the electron transport to nitrite

Membrane fragments prepared from *V. succinogenes* either with EDTA and lysozyme or with the French press were found to catalyze nitrite reduction by formate (reaction b) or H_2 (reaction c). Nitrate

$$3H_2 + NO_2^- + 2H^+ \rightarrow NH_4^+ + 2H_2O \quad (c)$$

reduction by these donors was not observed, although nitrate reductase as measured with reduced viologens was present.

Reaction (c) is catalyzed by an electron transport chain that involves MK as an obligatory component. This is shown by the experiment of Table 3. The extraction of more than 95% of the MK from the lyophilized membrane preparation caused a complete loss of the activity of reaction (c). The activity of fumarate reduction by H_2 was also abolished by this procedure. Addition of vitamin K_1, which is known to replace MK in electron transport (Kröger and Innerhofer 1976; Unden and Kröger 1982) to the preparation led to the reactivation of about 30% of the original activities of electron transport. The activity of hydrogenase as measured with benzyl viologen and that of nitrite reductase assayed with reduced benzyl viologen were found to be independent of the presence of MK.

Phosphorylation coupled to nitrite reduction

Membrane vesicles prepared with the French press catalyzed the phosphorylation of ADP as a function of reaction (c). Nitrite reduction by formate (reaction b) was not associated with phosphorylation in this preparation as was fumarate reduction by formate (Kröger and Winkler 1981).

The vesicles were incubated with limiting amounts of nitrite or fumarate and excess H_2 present, under conditions that allowed the phosphorylation of glucose in the presence of ADP and [^{32}P]phosphate. Organic phosphate was produced as a linear function of the amount of H_2 consumed (Fig. 3). From the slopes of the lines the P/H_2 ratios were evaluated as 0.11 with nitrite and 0.28 with fumarate as acceptor. Phosphorylation, but not electron transport was fully inhibited by low concentrations of the uncoupling agent FCCP (not shown).

Table 3. Electron transport activity to nitrite as a function of the MK content of the membrane fraction of *V. succinogenes*. The preparation of the membrane fraction, the extraction of MK, the reincorporation of vitamin K_1, the determination of MK (Kröger and Innerhofer 1976), measurement of the activity of fumarate reduction by H_2 (Kröger and Winkler 1981) and of benzyl viologen reduction by H_2 (Unden et al. 1982) were done as described. All the enzymic activities were measured at 37°C. The unit of enzymic activity is equivalent to the transport of 1 µmol H_2 per min

Membrane fraction	MK content (µmol/protein)	H_2 / NO_2^-	H_2 / Fumarate	H_2 / Benzyl viologen	Reduced benzyl viologen / NO_2^-
		(U/g protein)			
Lyophilized	4.8	570	1,640	600	19,540
Extracted	<0.1	4	4	480	9,330
Extracted +vitamin K	6	170	670	440	7,440

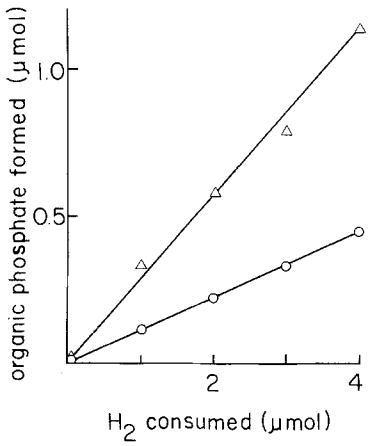

Fig. 3. Formation of organic phosphate as a function of electron transport with nitrite (○) or fumarate (△) as terminal acceptor. Phosphorylation was measured with membrane vesicles as described in the Methods section. The amount of H_2 consumed was calculated from the quantity of the acceptor added. The specific activities of H_2 oxidation by nitrite and fumarate were 0.16 and 1.1 µmol H_2/min per mg vesicle protein at 37°C

The lower P/H_2 ratio measured with nitrite cannot be explained by partial uncoupling by nitrite. In this case the P/H_2 ratio should decrease with increasing nitrite concentration, in contrast to the experimental result. The P/H_2 ratios measured suggest that the stoichiometric ATP gain of reaction (c) is about half that of fumarate reduction by H_2.

Discussion

Electron transport phosphorylation with nitrite as acceptor

It is demonstrated here for the first time that electron transport to nitrite is a quinone-dependent process. The electron transport chain in *V. succinogenes* is probably made up of the formate dehydrogenase complex (Kröger et al. 1979; Unden and Kröger 1982) or the hydrogenase complex

(Unden et al. 1982) and a nitrite reductase complex which are connected by MK. The nitrite reductase assayed with reduced benzyl viologen was extracted from the membrane fraction of *V. succinogenes* with Triton X-100 and purified (Roberton et al. unpublished). The enzyme was found to contain heme C and to be similar to the nitrite reductase of *Desulfovibrio desulfuricans* (Liu and Peck 1981). The electron transport chain may be similar to that of *Campylobacter sputorum* subsp. *bubulus*, which also catalyzes nitrite reduction to NH_4^+ by formate (de Vries et al. 1982). In this case the electron transport was inhibited by 2-(n-heptyl)-4-hydroxyquinoline-N-oxide (HQNO), and it was suggested that cytochromes are involved.

Evidence has been presented with other bacteria that nitrite reduction to NH_4^+ is associated with energy transduction (Cole 1978; de Vries et al. 1980, 1982; Motteram et al. 1981; Steenkamp and Peck 1981). However, electron transport phosphorylation coupled to nitrite reduction was never before demonstrated directly. Growth as a function of nitrite reduction by formate was first shown with *C. sputorum* subsp. *bubulus* (de Vries et al. 1980). The cell yields (Y) measured with a complex medium were about 4 g dry cells/mol formate with either nitrite, nitrate or fumarate.

ATP requirement for cell synthesis

Cell synthesis from succinate proceeds *via* fumarate in *V. succinogenes* growing with nitrate or nitrite as acceptor. The main biosynthetic pathways from fumarate with nitrate or nitrite appear to be identical to those used during growth with fumarate as terminal electron acceptor (Bronder et al. 1982). Succinate oxidation by nitrate is apparently not coupled to phosphorylation in *V. succinogenes*. This is concluded from the fact that this bacterium cannot grow on succinate and nitrate in contrast to others (Ohnishi 1963; Stouthamer 1976; van Verseveld et al. 1977; Kröger 1977; Thauer et al. 1977). As the oxidation of succinate by nitrate is catalyzed by resting cells (not shown), it is unlikely that the reaction requires ATP. Therefore, the ATP requirement for cell synthesis from succinate should be equal to that calculated earlier for biosynthesis from fumarate (55 mmol ATP/g dry cells, Bronder et al. 1982). The ATP requirement is not significantly altered by the presence of glutamate and cysteine as additional biosynthetic substrates.

ATP gain

The identical cell yields with nitrate and nitrite suggest that the ATP gain per mol formate is the same with both acceptors. The value of $Y^{max} = 8.5$ g dry cells/mol formate with nitrate or nitrite amounts to 60 % of that obtained earlier with fumarate as acceptor when the growth rate was limited catabolically (Mell et al. 1982). The growth rate was probably also limited by the energy metabolism with nitrate or nitrite as acceptor under the conditions of the experiment of Fig. 2. It is concluded, therefore, that the ATP gain of nitrate or nitrite reduction per mol formate is 60 % of that of fumarate reduction. The directly measured phosphorylation yield with nitrite and H_2 was 40 % of that accompanying fumarate reduction. The stoichiometric ATP gain with fumarate was measured earlier as 1 ATP/formate or H_2 (Kröger and Winkler 1981). It is concluded that the stoichiometric gain with nitrate or nitrite is approximately 0.5 ATP/formate or H_2.

The standard free energy differences per mol formate of reaction (a), (b) and (c) are nearly twice as negative as those of fumarate reduction by formate or H_2. It is, therefore, surprising that the stoichiometric ATP gain of the electron transport phosphorylation with nitrate or nitrite is lower than that with fumarate as acceptor in *V. succinogenes*. The reason for this discrepancy may reside in the mechanisms of energy transduction and of transport of nitrate and nitrite. Experiments on the accessibility of nitrate reductase and nitrite reductase by non-permeant redox dyes (not shown) suggest that both enzymes are oriented towards the inside of the cytoplasmic membrane. It is feasible that the electrochemical proton potential is generated by transmembrane electron transport as with fumarate (Kröger et al. 1980). However, while the transport of fumarate across the membrane does not require energy (Kröger et al. 1980), part of the energy of the proton potential generated with nitrate or nitrite may be used up by their uptake.

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