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Redox and specific effects of vanadium ions on respiratory enzymes

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The effects of vanadium ions on the activities of enzymes of aerobic and anaerobic respiratory chains were investigated *in vitro* and *in situ* employing ^{1}H-, ^{14}N-, ^{31}P- and^{51}V- nuclear magnetic resonance spectroscopy, electron paramagnetic resonance spectroscopy and spectrophotometry. Vanadate and vanadyl ions produced either non-specific redox or specific activation or inhibition of respiratory enzymes. The oxidants molybdate and chromate and the reductant dithiothreitol were used to distinguish between non-specific and specific effects of vanadium ions on enzyme activities. The results suggested that components of anaerobic respiratory chains were more susceptible to vanadium ions than those of the aerobic respiratory chain.

Keywords: vanadate; vanadyl; respiratoryenzymes; NMR spectroscopy; EPR spectroscopy.

Introduction

Many important physiological responses elicited by vanadium ions from living systems have been studied in detail during the last twenty years (Gresser & Tracey 1990). The large number of studies that have appeared recently reflect the complexity of these interactions, and the interest on vanadate and vanadyl ions owing to their environmental significance and potential medical applications. Two particular trends are noticeable in the new discoveries made on the effects of vanadium ions on biological systems: (a) the utilization of vanadium ions as probes to understand biochemical mechanisms, and (b) the investigation of their interactions with systems of increasing complexity.

On the other hand, only limited attention has been given to the effects of vanadate and vanadyl ions on respiration at the subcellular level. The effects of vanadium ions on mitochondria of different organisms have been studied (Desautels & Goldberg 1982, Nitschmann & Peschek 1984, Henderson *et al.* 1989, Zychlinski & Byczkowski 1990); and vanadate has been used also as a probe to analyze respiratory status (Desautels & Dulos 1993), metabolic control (Wisniewski *et al.* 1995), and calcium homeostasis (Gullapalli *et al.* 1989, Oz *et al.* 1992, Kunz *et al.* 1993). In particular, very few studies have been carried out to understand how vanadium affects individual components of respiratory systems, both isolated or *in situ.*

^{1}H-, ^{14}N-, ^{31}P- and ^{51}V- nuclear magnetic resonance (NMR) spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, and spectrophotometry were employed to investigate the effects of vanadium ions on enzymes involved in the electron transfer of aerobic and anaerobic respiration. The objective of this work was to establish (1) how these ions affected particular enzyme activities, (2) whether the observed effects were caused by vanadate or vanadyl ions, and (3) whether the effects, specific or non-specific, could be related to the type of respiratory chain in which the enzymes are found. Enzyme activities from prokaryote and eukaryote sources located at the beginning, middle and end of both types of respiratory chains were measured either *in situ* or in purified enzymes available commercially. The use of different sources helped to confer greater generality to the results.

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The results are presented in the sequence in which the enzyme activities are found in each respiratory chain. Firstly, hydroxybutyrate dehydrogenase, NADH:FMN dehydrogenase, transhydrogenase and cytochrome *c* reductase, for aerobic respiration. Secondly, formate dehydrogenase, nitrate reductases and fumarate reductase, for anaerobic respiration. Measurements were performed *in vitro* and *in situ* in buffers previously employed to observe the different enzyme activities. To distinguish whether vanadium ions produced specific or non-specific effects on enzyme activities, the actions of the metallate oxidants sodium molybdate (Na_2MoO_4) and sodium chromate (Na_2CrO_4), and the reductant dithiothreitol also were examined and compared with those of vanadate and vanadyl ions. The potentials of the three metallates follow the sequence: chromate > molybdate > vanadate.

Materials and methods

Substrates and reagents

Porcine heart cytochrome *c* reductase (EC 1.66.9.3), *Pseudomonas oxalaticus* formate dehydrogenase (EC 1.2.1.2), *Pseudomonas lemoignei* β-hydroxybutyrate dehydrogenase (EC 1.1.1.30), *Aspergillus* nitrate reductase (EC 1.6.6.2), acetoacetic acid lithium salt, dithiothreitol (DTT), oxidised flavin mononucleotide (FMN), fumarate, D-β-hydroxybutyrate, G-strophanthin (oubain), oxidised nicotinamide adenine dinucleotide (NAD^+) and reduced dinucleotide (NADH), and reduced nicotinamide adenine dinucleotide phosphate (NADPH), were obtained from Sigma (St Louis, MO). Equine heart oxidised cytochrome *c* was from Calbiochem (La Jolla, CA). Na_2CrO_4, Na_2MoO_4, NH_4VO_3 and $VOSO_4.3H_2O$ were from Aldrich (Milwaukee, WI). All other reagents were of analytical grade. The different buffers used for enzyme assays were taken from published reports.

Bacterial cultures and preparation

Campylobacter coli strain ATCC 33559 and *Campylobacter jejuni* strain 100 cells were grown on Blood Agar Base No. 2 plates supplemented with 5% (v/v) horse blood, polymyxin B (1.25 $U \cdot l^{-1}$), trimethoprim (5 $mg \cdot l^{-1}$), vancomycin (10 $mg \cdot l^{-1}$) and amphotericin B (2.5 $mg \cdot l^{-1}$), and incubated for 24 h in an atmosphere of 10% CO_2 in air, 95% humidity at 37 °C. Cell suspensions were prepared by harvesting cells in sterile NaCl (150 mM) and centrifuging them at $17{,}000 \times g$ (6 °C, 8 min), the supernatant was discarded and the pellet was collected and resuspended in NaCl. The procedure was repeated three times. Following the final wash, packed cells were resuspended to a concentration of approximately 10^8–10^9 $cells \cdot ml^{-1}$ in sterile NaCl. *Escherichia coli* strain K12 cells were grown aerobically on Luria-Bertani (LB) plates in air at 37 °C for 7 h, or anaerobically on LB plates supplemented with 20 mM $NaNO_3$ in jars with the gas generating anaerobic system BR 38 (Oxoid, Basingstoke,UK) at 37 °C for 10 h.

Bacterial lysates were prepared by harvesting cells in sterile NaCl (150 mM) and centrifuging them at $17{,}000 \times g$ (6 °C, 8 min), the supernatant was discarded and the pellet was collected and resuspended in NaCl. The procedure was repeated three times. Following the final wash, packed cells were resuspended to a concentration of approximately 10^8 $cells \cdot ml^{-1}$ in sterile KCl (150 mM). Lysates were prepared by either freeze-thawing cell suspensions twice in liquid nitrogen, or sonicating cell suspensions. Phase contrast microscopy indicated that more than 99% of the cells were lysed. Heat-denatured lysates were prepared by heating the samples at 80°C for 2 h in closed vials.

Nuclear magnetic resonancespectroscopy

For NMR measurements samples were placed into 5 or 10 mm tubes (Wilmad, Buena, NJ) and the appropriate substrates were added. Free induction decays were collected using a Bruker DMX-600 or ACP-300 NMR spectrometer, operating in the pulsed Fourier transform mode with quadrature detection. Measurements were carried out at 37 °C. One-dimensional ^{1}H-NMR spectra were acquired at 600.13 MHz with presaturation of the water resonance. The instrumental parameters were: spectral width 6009 Hz, memory size 16 K, acquisition time 1.363 s, number of transients 160, and relaxation delay with solvent presaturation 1.74 s. No window function was applied prior to Fourier transformation. ^{14}N-, ^{31}P- and ^{51}V-NMR spectra were acquired with composite pulse decoupling of protons. The instrumental parameters for observing ^{14}N were operating frequency 21.69 MHz, spectral width 10000 Hz, memory size 16 K, acquisition time 0.819 s, and pulse angle 90° (15μs). The number of transients was 1280 acquired with a standard Bruker antiring programme. Exponential filtering of 5 Hz was applied prior to Fourier transformation. The instrumental parameters for observing ^{31}P were: operating frequency 121.49 MHz, spectral width 3600 Hz, memory size 16 K, acquisition time 2.277 s, number of transients 200, and pulse angle 70° (15 μs). Exponential filtering of 2 Hz was applied prior to Fourier transformation. The instrumental parameters for observing ^{51}V were: operating frequency 78.95 MHz, spectral width 15000 Hz, memory size 16 K, acquisition time 0.541 s, number of transients 2500, and pulse angle 70° (15 μs). ^{51}V-NMR spectra were also acquired at an operating frequency of 131.47 MHz, with spectral width 26250 Hz, memory size 16 K, acquisition time 0.312 s, number of transients 2500, and pulse angle 70° (12 μs). Exponential filtering of 1 Hz was applied prior to Fourier transformation.

The time-evolution of metabolic reactions was followed by acquiring sequential spectra in which the resonances arising from substrates decrease in intensity and those corresponding to products increase in intensity with time.

Spinning of the samples ensured proper mixing throughout the experiments. The samples were kept in closed NMR tubes in an environment with reduced oxygen tension. Progress curves were obtained by measuring the integrals of substrate or product resonances at each point in time. Initial rates were calculated from good fits of the data to straight lines for the first 15–40 min of the reactions. Calibration of the peaks arising from substrates was performed by extrapolating the resonance intensity data to zero time and assigning to this intensity the appropriate concentration value. Calibration of the resonances arising from products was carried out by adding known concentrations of the corresponding metabolites to the samples.

Electron paramagnetic resonance spectroscopy (EPR)

Measurements were carried outusing a Bruker ESP-300 spectrometer. X-band spectra were obtained at 25 °C with an IR4102 cavity. The spectral width was 2000 gauss.

Spectrophotometry

Reduction rates of oxidised equine heart cytochrome *c* at 37°C were measured in a Cary 1 spectrophotometer. Oxidised cytochrome *c* was suspended in Tris-HCl (200 mM, pH 7.5) buffer and cytochrome *c* reductase added. In the absence of the enzyme the spectrum of oxidised cytochrome *c* did not change for at least one hour. Initial rates of reduction were calculated for the first 30 s of the reaction from the change in the absorbance at 411 or 550 nm corresponding to the γ and α electronic transitions of the reduced protein spectrum, respectively.

Effects of vanadate on bacterial growth in vitro

To investigate the effects of vanadate on cell growth, *E. coli* cells were grown aerobically or anaerobically on plates with different concentrations of NH_4VO_3 added. Twenty microlitres of *E. coli* cells suspended in isotonic NaCl to a density of approximately 10^5 cells/ml were inoculated and spread evenly over the surface of LB plates containing vanadate at concentrations between 0 and 1 mM. The solid media cultures were incubated either under aerobic conditions in air at 37 °C for 10 h, or under anaerobic conditions on plates supplemented with 20 mM $NaNO_3$ in glass jars at 37 °C for 20 h. Cells were harvested with a glass 'hockey stick' taking care to collect the entire culture, and washed three times in isotonic NaCl to remove proteins carried from the medium. Bacterial growth was measured by determining protein concentrations of suspensions of equal volumes (2 ml).

Protein determination

Protein estimation of samples was made by the bicinchoninic acid method employing a microtitre protocol (Pierce, Rockford, ILL).

Results

Interactions of vanadium ions with respiratory substrates and bacterial cell lysates

The interactions of NADPH, NAD^+, NADH, FMN, and bacterial lysates with NH_4VO_3 were investigated employing ^{1}H-, ^{31}P- and ^{51}V-NMR and EPR spectroscopy. The oxidation of NADPH or NADH in phosphate-KOH (66 mM, pH 7) buffer was measured by the decline with time of the ^{1}H-2 and ^{1}H-8 resonances of the adenine moiety employing ^{1}H-NMR spectroscopy. These resonances have different chemical shifts in the reduced and oxidised dinucleotides; thus upon oxidation of NADH or NADPH the ^{1}H-2 and ^{1}H-8 resonances of the adenine moiety disappear from the spectrum and concomitantly the corresponding resonances of NAD^+ and $NADP^+$ appear at different spectral positions (Fig. 3). At 20 mM concentration of either dinucleotide rates of 17 ± 2 and 5 ± 1 nmole·min^{-1} were determined with and without 2 mM NH_4VO_3, respectively. Rates of chemical reduction of vanadate in HEPES buffer were measured by the decrease in the resonances of monomeric, dimeric and tetrameric vanadate (Crans *et al.* 1990) with time (Fig. 1). Reduction rates of 3.5 ± 0.5 nmole·min^{-1} were determined for solutions of 2 mM NH_4VO_3 and 20 mM NADPH or NADH in HEPES-KOH (50 mM, pH 7) buffer. The rates measured for the vanadate monomer, dimer and tetramer were 0.4 ± 0.04, 0.5 ± 0.05 and 2.6 ± 0.3 nmole·min^{-1}, respectively. The metavanadate/dinucleotide solutions acquired a pale yellow-gray colour with time suggesting the formation of vanadyl ions. The nature of the products of these reactions was investigated employing EPR spectroscopy. The EPR spectra indicated that vanadate (VO_3^-) was partially reduced to vanadyl (VO^{2+}) in NADH:NH_4VO_3 (8:1, mol/mol) solutions in HEPES-KOH buffer. The rates of oxidation of both dinucleotides in HEPES buffer were not affected by vanadyl sulfate at concentrations up to 1 mM. No significant reduction of vanadate was observed for 450 min in a solution containing 2 mM NH_4VO_3, 5 mM NAD^+ and 10 mM NADPH.

The ^{51}V-NMR resonances of 2 mM NH_4VO_3 in Tris-HCl (50 mM, pH7.4), 0.2 mM EDTA buffer were not affected by addition of 10 mM FMN; and the ^{31}P-NMR spectrum of 10 mM FMN in the same buffer did not change by addition of 2 mM NH_4VO_3 or 2 mM $VOSO_4$, indicating that there were no interactions between the vanadium ions and the mononucleotide. Under the same conditions, chemical oxidation of 20 mM NADH or NADPH in the

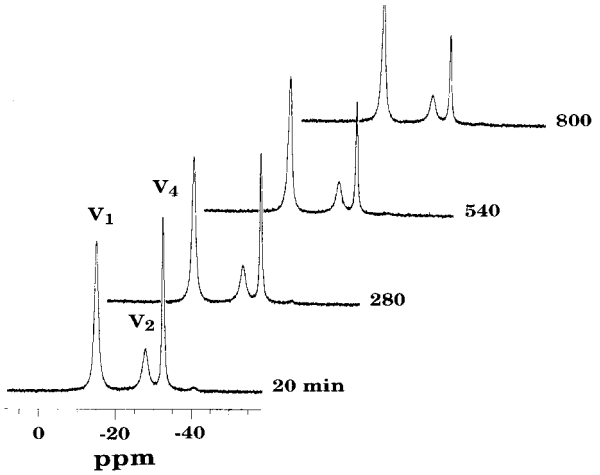

Figure 1. Sequential ^{51}V-NMR spectra of NH_4VO_3
Segment of the 131.5 MHz ^{51}V-NMR spectra of a 2 mM NH_4VO_3, 20 mM NADH in HEPES-KOH (50 mM, pH 7) buffer solution at 310 K. The resonances arising from monomeric (V_1), dimeric (V_2) and tetrameric (V_4) vanadate are labelled (Crans *et al.,* 1990). The time at which each spectrum was acquired is indicated on the right-hand side. The spectra are referenced with respect to a 3 mM vanadate, pH 14 capillary at 0 ppm.

presence of 10 mM FMN was observed using ^{31}P-NMR spectroscopy, and was not affected by the addition of 2 mM $VOSO_4$. The fate of vanadate in Tris-HCl buffer solutions containing either dinucleotide and FMN at the above concentrations was investigated with ^{51}V-NMR and EPR spectroscopy. The resonances of 2 mM NH_4VO_3 disappeared from the ^{51}V-NMR spectrum within 3 min; and the EPR spectrum showed that vanadate was reduced to vanadyl (Fig. 2).

The effects of 2 mM dithiothreitol added to 2 mM NH_4VO_3 solutions in HEPES-KOH (50 mM, pH 7.2), 1 mM $MgCl_2$, 0.2 mM EDTA or Tris-HCl (50 mM, pH 7.4), 0.2 mM EDTA buffers were investigated with ^{51}V-NMR spectroscopy. After adding DTT the resonance of the vanadate monomer at –14.8 ppm was unchanged, but within minutes the intensities of the resonances at –29.3 and –33.9 ppm corresponding to vanadate dimers and tetramers, respectively (Fig. 1), were significantly diminished, and resonances at 19.5 and 86.5 ppm corresponding to decavanadate appeared in the spectrum. After these initial changes the ^{51}V-NMR spectrum remained unaltered for 24 h.

Within minutes after adding 10 mg of equine heart oxidised cytochrome*c* and 2.5 mM NADH the intensities of the ^{51}V-NMR monomer, dimer and tetramer resonances of 2 mM NH_4VO_3 in Tris-HCl (200 mM,

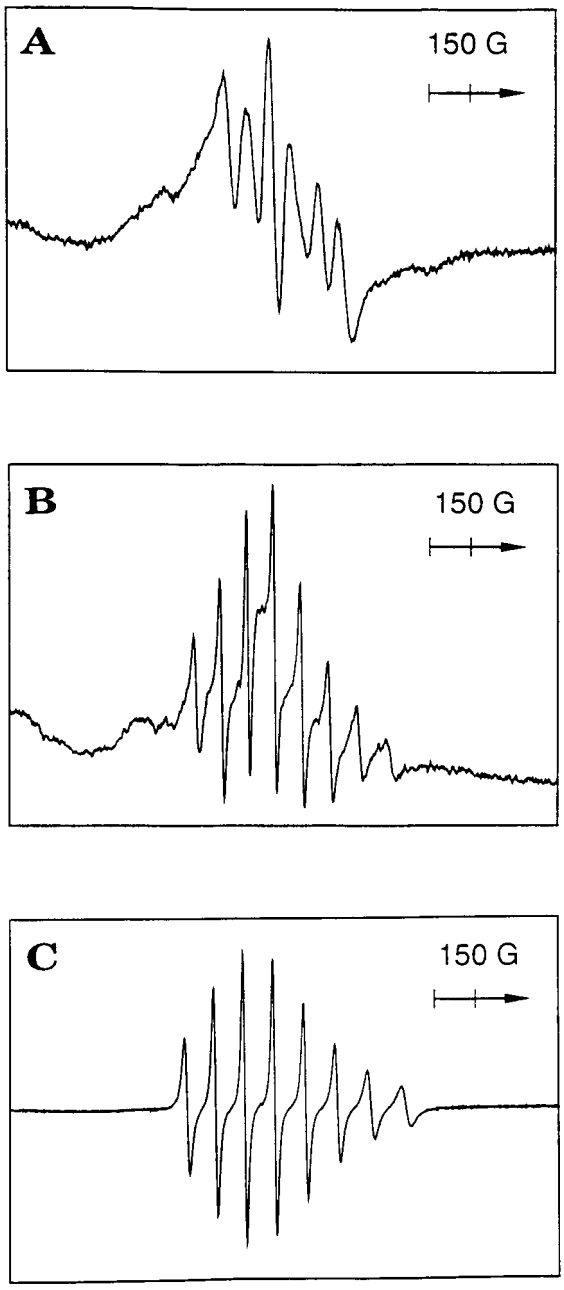

Figure 2. EPR spectra of vanadyl ions formed by interactions of vanadate with respiratory substrates and formate dehydrogenase
X-Band EPR derivative spectra at 298 K of (A) 2 mM NH_4VO_3, 10 mM FMN, 20 mM NADH in Tris-HCl (50 mM; pH 7.4) buffer; (B) 10 mM NH_4VO_3, 1.5 mg *P. oxalaticus* formate dehydrogenase in phosphate-KOH (50 mM, pH 7) buffer; and (C) 5 mM VO_2SO_4 in aqueous solution. The latter spectrum shows the eight-line hyperfine structure characteristic of the unpaired electron strongly coupled to the ^{51}V nucleus, also present in spectra (A) and (B). The magnetic field scale and direction are shown on the upper right-hand corner of each panel.

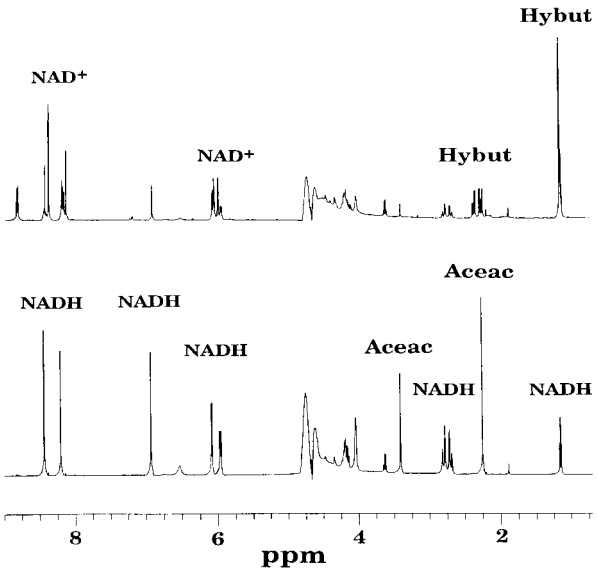

Figure 3. ^{1}H-NMR spectra of substrates and products of the reaction catalysed by hydroxybutyrate dehydrogenase *Bottom:* 600 MHz ^{1}H-NMR spectrum of 10 mM acetoacetate (Aceac), 10 mM NADH and 0.05 U·ml^{-1} of *P. lemoignei* hydroxybutyrate dehydrogenase in phosphate-KOH (66 mM, pH 7), 15 mM KCl buffer at 310 K. *Top:* spectrum of the products hydroxybutyrate (Hybut) and NAD^+ of the dehydrogenase reaction after 24 h incubation. The spectra are referenced to TMS at 0 ppm.

pH 7.5) decreased by 25, 30 and 30%, respectively, and resonances arising from decavanadate were observed at 22.5 ppm as previously reported for other proteins (Rehder, 1990). No further changes were observed in the spectrum for 240 min.

Addition of *C. coli, C. jejuni* or *E. coli* bacterial lysates at concentrations up to 4 mg·l^{-1} did not produce any changes in the ^{51}V spectrum of 2 mM NH_4VO_3 in HEPES-KOH (50 mM, pH 7) buffer, suggesting that no redox interactions occurred between vanadate and the lysates.

Hydroxybutyrate dehydrogenase

The activity of *P. lemoignei* hydroxybutyrate dehydrogenase (EC 1.1.1.30) was measured employing ^{1}H-NMR spectroscopy in suspensions of the enzyme in phosphate-KOH (66 mM, pH 7), 15 mM KCl buffer. Rates were measured in the forward direction by the oxidation of hydroxybutyrate by NAD^+, and in the reverse direction by the reduction of acetoacetate by NADH (Fig. 3). Validation of the method was performed by leaving out NAD^+ or NADH and observing no oxidation of hydroxybutyrate or reduction of acetoacetate, respectively. In the absence of the enzyme no redox reactions between the appropriate substrates were observed for 60 min. Hydroxybutyrate dehydrogenase activities measured in the reverse direction by the decline in the levels of acetoacetate were linear with the amount of enzyme up to 0.15 U.ml^{-1} and 15 mM substrate concentrations. In suspensions containing 0.05 U of the dehydrogenase, and substrate concentrations up to 15 mM, enzyme activities were linear for 40 min. The rate of reduction of acetoacetate measured for the first 20 min at substrate concentrations of 10 mM was 800 ± 65 nmole·min^{-1}·U^{-1} (n = 3), and decreased by less than 3 ± 2% in the presence of 1 mM vanadate (n = 3). Addition of 1 mM molybdate or chromate decreased acetoacetate rates of reduction by 12 ± 2% and 13 ± 2%, respectively (n=3), indicating that the metallates did not affect significantly hydroxybutyrate dehydrogenase activity. At 10 mM concentrations of hydroxybutyrate and NAD^+ the rates of oxidation of hydroxybutyrate in the forward direction were not affected by the presence of any of the three the metallates at 1 mM concentrations. DTT at 1 mM concentration did not produce any significant increase in the forward or reverse rates.

The interactions of hydroxybutyrate dehydrogenase with NH_4VO_3 were investigated also employing EPR spectroscopy. No reduction of vanadate was observed in a suspension containing 10 mM NH_4VO_3 and 1.5 mg of hydroxybutyrate dehydrogenase in phosphate-KOH buffer, suggesting that there were no direct enzyme-vanadate redox reactions.

NADH:FMN dehydrogenase

The effects of vanadate on *E. coli* respiratory NADH:FMN dehydrogenase (EC 1.6.99.3) were studied *in situ* in lysates prepared from cells grown aerobically and suspended in Tris-HCl (50 mM, pH 7.4), 0.2 mM EDTA buffer. The oxidation of NADH was measured using ^{31}P-NMR spectroscopy by the decline in the reduced dinucleotide concentration and the appearance of NAD^+ for the first 15 min of the reaction. No significant oxidation of NADH above the background occurring in aqueous suspensions was observed in lysate suspensions in the absence of FMN. Observed rates of dinucleotide oxidation with 10 mM NADH and 5 mM FMN were linear with substrate concentrations, and at lysates concentrations between 0.5 and 2 (mg protein)·ml^{-1} of lysate.

NADH:FMN dehydrogenase net rates were determined by subtracting the rates of chemical oxidation of 10 mM NADH by 5 mM FMN in heat-denatured lysates from the values measured for normal lysates.

Addition of 1 mM NH_4VO_3 or $VOSO_4$ did not change the rates of oxidation of NADH by FMN in heat-denatured lysates. The rate of *chemical oxidation* was decreased by 30% in presence of 1 mM DTT, and increased by 40% in the presence of 1 mM Na_2CrO_4. The rate of NADH:FMN dehydrogenase measured in lysates with 10 mM NADH and 5 mM FMN at 37 °C was 48 ± 5 nmole min^{-1} mg^{-1} (n = 3). In the presence of 1 mM vanadate or vanadyl the enzymic rate was 35 ± 4 nmole $min^{-1} \cdot mg^{-1}$ (n = 4). Dehydrogenase rates measured in the presence of 1 mM DTT or chromate were 71 ± 8 and and 28 ± 3 nmole $\cdot min^{-1} \cdot mg^{-1}$, respectively (n = 3) (Table 1). No effects on NADH:FMN dehydrogenase activity were observed in the presence of 1 mM of the alkylating agent iodoacetamide or 1 mM of the non-specific sulfhydryl *p*-chloromercuribenzoate.

Table 1. Effects of Vanadium Ions, Chromate and DTT on the Rates of NADH:FMN Dehydrogenase in *E. coli* K12 Lysates

Compound	Measured rate (nmole min^{-1})	Enzymic rate (nmole min^{-1} mg^{-1})
Control	841 ± 85	48 ± 5
Vanadate	635 ± 67	35 ± 4
Vanadyl	645 ± 67	35 ± 4
Chromate	834 ± 82	28 ± 3
DTT	706 ± 77	71 ± 8

NADH:FMN dehydrogenase net rates were determined by subtracting the rates of chemical oxidation of 10 mM NADH by 5 mM FMN in heat-denatured lysates from the values measured for normal lysates.The measured rate includes the chemical and enzymic rates of NADH oxidation. Three independent measurements were carried out for each set of conditions.

Transhydrogenase

The effect of metallates on the transfer of two reducing equivalents from NADPH to NAD^+ in the reaction catalysed by transhydrogenase (EC 1.6.1.1) was studied employing ^{31}P-NMR spectroscopy. *E. coli* lysates of bacteria grown aerobically were suspended in HEPES-KOH (50 mM, pH 7.4), 0.33 mM EDTA, 7 mM $MgCl_2$, 25 mM KCl buffer, and incubated with NADPH and NAD^+. Rates of reduction of NAD^+ by NADPH were measured by the appearance of NADH. In the absence of NADPH, no NADH product was formed. Transhydrogenase activities were determined by subtracting the values of the rates of chemical reduction of NAD^+ from the values measured with the lysates added. At concentrations of 5 mM NADPH and 2.5 mM NAD^+ and lysate concentrations between 1 and 4 (mg protein)$\cdot ml^{-1}$, the reaction was linear for at least 90 min, and rates were determined for the first 40 min. The measured rate of chemical reduction of NAD^+ was 7.7 ± 0.7 nmole min^{-1} (n = 3) at 5 mM NADPH and 2.5 mM NAD^+ concentrations. This rate was not altered by the presence of NH_4VO_3, Na_2MoO_4, Na_2CrO_4 or DTT up to 1 mM concentrations; the presence of $VOSO_4$ increased the rate of production of NADH by 30%. The enzyme activity was linear with both substrates at concentrations of 5 mM NADPH and 2.5 mM NAD^+; the rate measured under these conditions was 2.0 ± 0.3 nmole $\cdot min^{-1} \cdot mg^{-1}$ (n = 3). Transhydrogenase activity increased in the presence of vanadate or vanadyl, at 1 mM concentrations the increases were $55 \pm 6\%$ and $82 \pm 8\%$, respectively (n = 2). The presence of 1 mM DTT did not alter the rate of enzyme activity, and 1 mM Na_2MoO_4 or Na_2CrO_4 decreased transhydrogenase rates to 1.4 ± 0.2 and 0.8 ± 0.1 nmole $min^{-1} \cdot mg^{-1}$, respectively (n = 2).

Cytochrome c *reductase*

Porcine heart cytochrome *c* reductase (EC 1.6.99.3) activities were measured spectrophotometrically. The rates of cytochrome *c* reduction were linear with enzyme concentrations between 0.03 and 0.1 $U \cdot ml^{-1}$, and substrate concentrations of 0.1 to 0.5 $mg \cdot ml^{-1}$ oxidised heart cytochrome *c* and 0.5 to2 mM NADH. The activity of the enzyme was measured with or without NADH, and a 3-fold increase in activity was observed at saturating concentrations (5 mM) of the dinucleotide. Measurements carried out with 0.08 $U \cdot ml^{-1}$ cytochrome *c* reductase, 0.3 $mg \cdot ml^{-1}$ oxidised heart cytochrome *c,* and 1 mM NADH showed a linear increase in enzyme activity in the presence of NH_4VO_3 up to a concentration of 80 μM; with a 50% activation at 40 ± 8 μM, with or without NADH as cosubstrate. Under these conditions increases in the concentration of the oxyanion produced further stimulation of the enzyme, and at 200 μM metavanadate cytochrome *c* reductase activity was enhanced 3-fold (n = 3). Both Na_2MoO_4 and Na_2CrO_4 produced concentration-dependent decreases of enzymic rates which depended on the presence of NADH as cosubstrate. At 200 μM Na_2MoO_4 or Na_2CrO_4 the rates had decreased to 80% and 70% with NADH, and 60% and 50% without NADH, respectively. Oxidised cytochrome*c* was reduced chemically by DTT. At enzyme concentrations between 0.04 and 0.08 $U \cdot ml^{-1}$ the observed reduction rates were smaller than the addition of the measured enzymic and chemical rates of reduction, suggesting that the enzyme was inhibited by DTT.

Formate dehydrogenase

P. oxalaticus formate dehydrogenase (EC 1.2.1.2) activities were determined by measuring initial rates of decline of formate levels employing ^{1}H-NMR spectroscopy (Fig. 4). Enzyme activity was measured in preparations with 0.5 to 1.4 U·ml^{-1} of enzyme suspended in phosphate-KOH (50 mM, pH 7) buffer with 25 mM formate and 20 mM NAD^+. Under these conditions the reaction was linear for 25 min, and the rate measured was 152 ± 13 nmole·min^{-1}.mg^{-1} (n = 3). No decrease of formate concentration was observed in the absence of the enzyme or NAD^+. The presence of vanadate inhibited the enzyme, with an IC_{50} of 30± 5 μM NH_4VO_3. Addition of DTT (200μM) to the assay mixtures prevented inhibition of formate dehydrogenase by metavanadate at concentrations up to 500 μM. The presence of 30 μM Na_2MoO_4 or Na_2CrO_4 resulted in a decline of 15 ± 2% of the initial rates of formate dehydrogenase.

The interactions of *P. oxalaticus* formate dehydrogenase with NH_4VO_3 were investigated employing EPR spectroscopy. Vanadate was reduced to vanadyl by the enzyme; in suspensions containing 10 mM NH_4VO_3 and 1.5 mg of formate dehydrogenase, 0.30 ± 0.06 mM VO_2^{2+} were produced (Fig. 2), suggesting that groups in the enzyme became oxidized by the metallate.

Nitrate reductases

^{14}N-NMR spectroscopy was employed to measure the reduction of nitrate by nitrate reductase (EC 1.6.6.2) from *Aspergillus* sp. in the presence of NADPH. Nitrate is observable with ^{14}N-NMR spectroscopy owing to the symmetry of the chemical group; reduction to nitrite makes the signal invisible, thus reduction of nitrate was measured by the decrease of its ^{14}N resonance. Enzyme suspensions were prepared in HEPES-KOH (50 mM, pH 7.4), 10 mM K_2HPO_4, 10 mM NaCl, 10 mM KCl buffer. Under these conditions there was no chemical reduction of nitrate in the presence of NADPH, and nitrate was not reduced enzymically in the absence of NADPH. At initial 15 mM $NaNO_3$ and NADPH concentrations and enzyme activity of 0.7 U·ml^{-1}, the reduction of nitrate was linear with the concentrations of both substrates for 90 min. The rates of nitrate reduction measured during the first 50 min of the reaction decreased linearly with ammonium vanadate concentration with an IC_{50} of 148 ± 10 μM NH_4VO_3. Molybdate and chromate also inhibited nitrate reductase, but DTT activated the enzyme (Table 2).

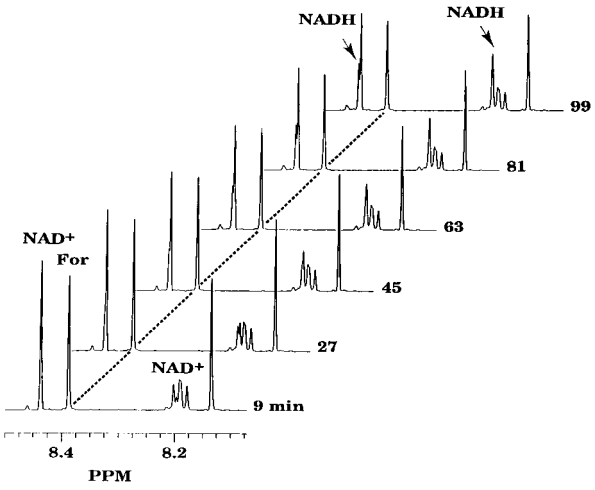

Figure 4. Sequential ^{1}H-NMR spectra of substrates and products of the reaction catalysed by formate dehydrogenase

Segment of the 600 MHz ^{1}H-NMR spectra of 25 mM formate, 20 mM NAD^+, 0.5 U·ml^{-1} of *P. oxalaticus* formate dehydrogenase in phosphate-KOH (50 mM, pH7) buffer at 310 K. Resonances arising from the substrates formate (For) and NAD^+ and the product NADH are labelled on the bottom spectrum. Resonances arising from the product NADH are labelled on the top spectrum. The dashed line follows the decline of the formate resonance with time. The time at which each spectrum was acquired is indicated on the right-hand side. The spectra are referenced to TMS at 0 ppm.

Table 2. Effects of Metallates and DTT on the Rates of Nitrate Reduction by *Aspergillus* Nitrate Reductase (NADPH) and *E. coli* K12 Nitrate Reductase (NADH) Activity

Compound	*Aspergillus* rate (nmole min^{-1} mg^{-1})	*E. coli* rate (nmole min^{-1} mg^{-1})
Control	99 ± 11	160± 20
IC_{50}	67 ± 8	111 ± 13
Molybdate	51 ± 6	84 ± 10
Chromate	17 ± 2	37 ± 4
DTT	198 ± 21	200 ± 22

Initial substrate concentrations were 15 mM $NaNO_3$ and 15 mM NADH. The concentrations of metallates and DTT were 0.15 and 0.5 mM for the *Aspergillus* enzyme and *E. coli* activity, respectively. Three independent measurements were carried out for each set of conditions.

No reduction of vanadate to vanadyl was observed employing EPR spectroscopy in suspensions of 1.5 mg *Aspergillus* nitrate reductase and 10 mM NH_4VO_3 in HEPES-KOH (50 mM, pH 7.4), 10 mM K_2HPO_4, 10 mM NaCl, 10 mM KCl buffer.

The effects of vanadate on *E. coli* nitrate reductase (EC 1.6.6.1) were investigated using ^{14}N-NMR spectroscopy in lysates prepared by sonication of cells grown anaerobically, and suspended in Tris-HCl (50 mM, pH 7.5), 75 mM KCl buffer. No chemical reduction of nitrate by NADH was observed in the absence of lysates, and nitrate was not reduced enzymically in the absence of NADH. At 15 mM $NaNO_3$ and NADH the rates of nitrate reduction were linear with substrate concentrations and lysate concentration up to 2 mg·l^{-1}. Under these conditions nitrate reduction was linear for 70 min and rates of 160 ± 20 nmole·min^{-1}·mg^{-1} were measured during the first 40 min of the reaction. The rates of NADH reduction decreased with metavanadate concentration, and the estimated IC_{50} was 1.1 ± 0.2 mM NH_4VO_3.

Addition of DTT to the assay mixtures enhanced *E. coli* nitrate reductase; the enzyme activity was 200 ± 22 nmole·min^{-1}·mg^{-1} in the presence of 0.5 mM DTT. Na_2MoO_4 and Na_2CrO_4 inhibited *E. coli* nitrate reductase and the strength of the effect increased with the redox potential of the metallates (Table 2).

Fumarate reductase

Fumarate reductase (EC 1.3.99.1) activity was measured in intact *C. jejuni* cells suspended in isotonic saline and in *C. coli* lysates suspended in phosphate-KOH (66 mM, pH 7), 60 mM KCl buffer employing a ^{1}H-NMR method previously described (Mendz, *et al.,* 1995). A rate of succinate production of 22.7 ± 1.6 nmole·min^{-1}·mg^{-1} was measured at 10 mM fumarate concentration for *C. jejuni* cells (n=3). Rates decreased linearly with NH_4VO_3 concentration, and an IC_{50} of 1.2 ± 0.1 mM vanadate was determined for *C. jejuni* fumarate reductase.

In *C. coli* lysates the rate of succinate production was 10.5 ± 0.9 nmole·min^{-1}·mg^{-1} (n = 3). The presence of 1 mM NH_4VO_3, Na_2MoO_4 or Na_2CrO_4 produced 30%, 35% and 45% inhibition of fumarate reductase activity, respectively. Addition of 1 mM DTT resulted in 20% *increase* in the rate of succinate production in the lysates. Addition of both 1 mM NH_4VO_3 and 1 mM DTT resulted in a non-significant decrease in fumarate reductase activity of 5 ± 3%.

Effects of vanadate on bacteria grown aerobically and anaerobically

Inhibition of *E. coli* cell growth was observed in plates supplemented with vanadate up to 1 mM concentration. In the absence of vanadate growth of 11.97 ± 0.06 and 8.94 ± 0.06 (mg protein)·ml^{-1} were measured under aerobic and anaerobic conditions, respectively. In the presence of 1 mM vanadate the aerobic and anaerobic cell growth measured were 10.11 ± 0.06 and 6.06 ± 0.06 (mg protein)·ml^{-1}, respectively. The inhibition of growth increased linearly with the concentration of vanadate in the cultures and the inhibitory effect was twice as strong on cells grown anaerobically (Fig. 5). Vanadate can inhibit also enzymes involved in phosphoryl transfer reactions. In particular ATPases are susceptible to the action of the metallate at micromolar concentrations (Gresser & Tracey, 1990). To ascertain whether these different effects of vanadate on cells resulted principally from the action of the metallate ions on ATPases, a control experiment was performed including G-strophanthin, a potent ATPase inhibitor. The effects of vanadate (0–400 μM), G-strophanthin (0–200 μM) and vanadate with G-strophanthin were measured on *E. coli* cells grown aerobically and anaerobically. The inhibition of growth by vanadate was as reported above. The inhibition by G-strophanthin was linear with inhibitor concentration, and affected equally cells grown aerobically or anaerobically. The inhibition by vanadate and G-strophanthin was about 60% stronger for cells grown anaerobically than for cells grown aerobically.

A summary of the effects of vanadium ions on different enzyme activities of aerobic and anaerobic respiratory chains, including the measured IC_{50} and the mode of action is given in Table 3.

Discussion

Vanadium ions produced different effects on the activities of enzymes that operate at the beginning of aerobic and anaerobic respiratory chains: hydroxybutyrate dehydrogenase and formate dehydrogenase, respectively (Table 3). *P. lemoignei* hydroxybutyrate dehydrogenase was not affected by the presence of NH_4VO_3, Na_2MoO_4 or Na_2CrO_4 up to 1 mM concentrations. On the other hand, vanadate produced a strong inhibition of *P. oxalaticus* formate dehydrogenase, which was prevented by the presence of DTT in the samples, suggesting that the enzyme may have been inactivated through oxidation by vanadate. However, the presence of the stronger oxidants molybdate and chromate induced only 15% inhibition of enzyme activity, indicating that non-specific oxidation of formate dehydrogenase would result only in partial inactivation during the time of the assay; thus oxyvanadium ions pro-

Table 3. Summary of effects of vanadium ions on different enzyme activities of aerobic and anaerobic respiratory chains, including the measured IC_{50} and the mode of action.

Enzyme	Ion	IC_{50} (μM)	Mode of action
Hydroxybutyrate Dehydrogenase	–	–	No effect
NADH:FMN Dehydrogenase	Vanadyl	ND	Redox inhibition
Transhydrogenase	Vanadyl	610 ± 60	Specific activation
Cytochrome *c* Reductase	Vanadate	40 ± 8	Specific activation
Formate Dehydrogenase	Vanadate	30 ± 5	Specific inhibition
Nitrate Reductase (NADPH)	Vanadate	148 ± 10	Redox inhibition
Nitrate Reductase (NADH)	Vanadate	1100 ± 200	Redox inhibition
Fumarate Reductase	Vanadate	1200 ± 100	Redox inhibition

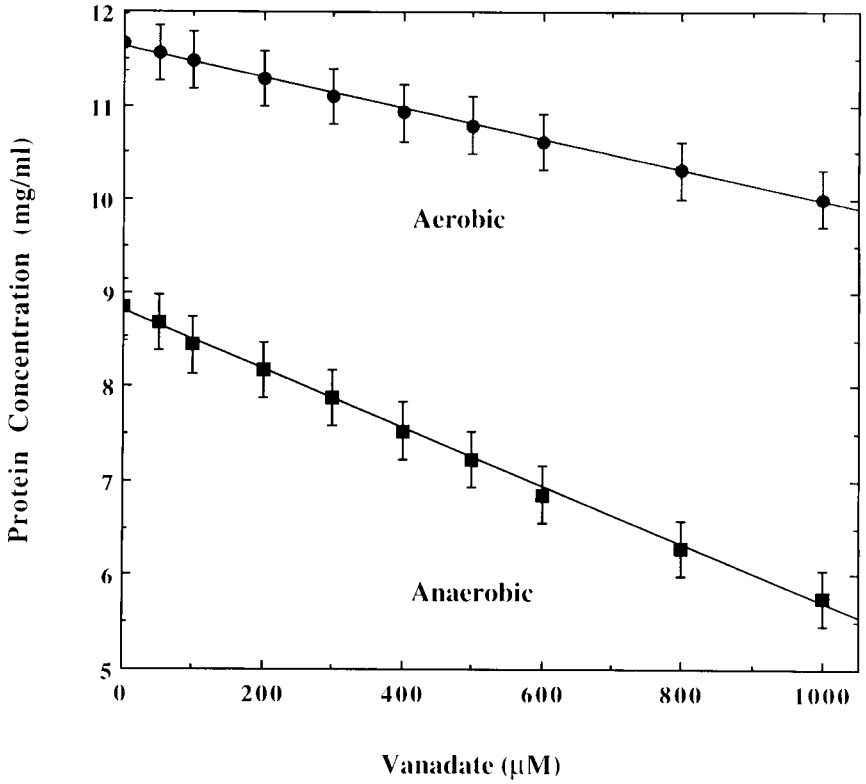

Figure 5. Growth inhibition of *E. coli* K12 on plates supplemented with NH_4VO_3
E. coli bacteria were grown on LB plates under aerobic or anaerobic conditions at 310 K for 20 h. The plates were supplemented with different concentrations of NH_4VO_3. Bacterial growth measured by the protein content of each plate is plotted as a function of the concentration of vanadate in the plates. The inhibition of growth was linear with oxyvanadium concentration, and the metallate had an inhibitory effect twice as strong on cells grown anaerobically.

duced specific inhibition of formate dehydrogenase. A mechanism proposed for the inhibition of glyceraldehyde-3-phosphate dehydrogenase by vanadate was the oxidation of sulfhydryl groups in the enzyme with a reduction of vanadate to vanadyl and the formation of oxovanadium-protein complexes (Benabe *et al.* 1987). Reduction of vanadate to vanadyl by formate dehydrogenase was observed employing EPR spectroscopy (Fig. 2). Considering that the effects of the metallate were reversed by the sulfhydryl-reducing agent DTT, it can be concluded that vanadate affects formate dehydrogenase by interacting with sulfhydryl groups involved in enzyme activity.

In aqueous buffer solutions vanadyl ions are generated from vanadate in a Fenton-like reaction by O_2^--dependent oxidation of the dinucleotides NAD(P)H (Shi & Dalal 1993). Vanadate was also reduced in redox reactions involving the oxidation of NAD(P)H by another nucleotide, such as those catalysed by NADH:FMN dehydrogenase and trans-

hydrogenase. NADH:FMN dehydrogenase rates in *E. coli* lysates decreased equally by the presence of NH_4VO_3 and $VOSO_4$ (Table 1); since fast chemical reduction of VO_3^- to VO^{2+} occurred in the presence of NADH and FMN (Fig.2), these results indicated that vanadyl was the vanadium ion responsible for the decrease in enzyme activity. To establish whether this effect was specific to vanadium ions or depended on the redox conditions, the activity of the enzyme was measured in the presence of the reductant DTT, and was found to increase by 48% (Table 1). Since the activity was not changed by iodoacetamide and *p*-chloromercuribenzoate, the data suggested that sulfhydryl groups were not involved in the functioning of the enzyme, and the effects of DTT on NADH:FMN dehydrogenase were ascribed to its properties as a reductant. This conclusion was supported by the observation that the dehydrogenase activity decreased in the oxidizing environment produced by the presence of the oxidant Na_2CrO_4. Transhydrogenase is another enzyme catalysing a redox reaction between nucleotides. In *E. coli* lysates this activity was stimulated *in situ* 55% and 82% by NH_4VO_3 and $VOSO_4$, respectively. Since dithiothreitol did not alter transhydrogenase rates, and chromate decreased them, it was concluded that the observed effects were due to oxovanadium ions.

Although NADH dehydrogenases that use vanadate as electron acceptor have been reported (Ramasarma *et al.* 1981), the control experiments with *E. coli* NADH:FMN dehydrogenase and transhydrogenase indicated that this was not the case for the bacterial enzyme activity. Two mechanisms have been proposed to account for the increase in NADH oxidation rates by vanadate: a free-radical chain oxidation initiated by the reaction of radicals produced by the activity of the enzyme with vanadate as an oxidant, and a direct activation of the enzyme itself by vanadate (Reif *et al.* 1989), but the data of that study did not permit to differentiate between both mechanisms. In the present work it has been found that vanadyl was the vanadium ion specifically inhibiting NADH:FMN dehydrogenase and enhancing transhydrogenase activities, suggesting mechanisms of direct enzyme activation by oxovanadium rather than free-radical chain oxidations with vanadate as an oxidant. Cytochrome *c* reductase was activated by the presence of vanadate and inhibited by molybdate and chromate, whether NADH was a cosubstrate or not. Although interactions of NH_4VO_3 with enzymes can generate superoxide ions known to be strong reductants of cytochrome *c*, the stimulation of cytochrome *c* reductase by vanadate ions was interpreted as a direct interaction with the enzyme, because the stronger oxidants Na_2MoO_4 and Na_2CrO_4 were inhibitory.

The effects of vanadium on the activities of electron carriers involved in aerobic or anaerobic respiration were qualitatively different (Table 3). Transhydrogenase, cytochrome *c* reductase, nitrate reductase (NADPH) and nitrate reductase (NADH) are enzymes which employ NAD(P)H to reduce different substrates; the first two are part of the aerobic respiratory chain and the last two are part of anaerobic respiratory chains. All four activities decreased in the presence of the oxidants Na_2MoO_4 and Na_2CrO_4; but the activities of the aerobic enzymes were enhanced in the presence of vanadium ions, whereas vanadate inhibited the activities of the anaerobic enzymes. The reductant DTT enhanced the anaerobic enzyme activities (Table 2); but it did not affect transhydrogenase activity, and inhibited cytochrome *c* reductase. The results suggested that vanadium ions had specific activation effects on the aerobic enzymes, and only redox inhibitory effects on the nitrate reductases (Table 3).

The activity of anaerobic fumarate reductase in *C. jejuni* cells decreased linearly with NH_4VO_3 concentration. To understand the nature of this inhibition, the effects of vanadate, molybdate and chromate on the activity of the enzyme in *C. coli* lysates were compared. The greater inhibition caused by the stronger oxidants molybdate and chromate, suggested that the effects observed with oxyvanadium arose from oxidation of the enzyme rather than from specific interactions with vanadate ions. Evidence supporting this conclusion was that the presence of DTT increased fumarate reductase activity by 20%, and equimolar concentrations of the reducing reagent reversed the effects of vanadate.

E. coli switches its mode of respiration from aerobic to anaerobic when oxygen is removed from the incubation atmosphere. In cultures supplemented with nitrate, this anion becomes the terminal electron acceptor in respiration instead of oxygen, and there is concomitant expression of the enzymes characteristic of nitrate respiration and suppression of those characteristic of oxygen respiration. The results measured for *E. coli* cells grown under aerobic and anaerobic conditions in the presence of different concentrations of NH_4VO_3 indicated a greater susceptibility to vanadate in cells grown anaerobically. Through its phosphate-mimicking effects vanadate is an inhibitor of P-type ATPases, phosphatases, kinases and several ATP-Binding Cassette transporters (Hunke*et al.* 1995). The data comparing the effects on cell growth of vanadate, G-strophanthin, and vanadate with G-strophanthin

indicated that the effects observed with vanadate alone did not arise principally from inhibition of ATPases. Although the greater inhibition of anaerobic cell growth by vanadate cannot be ascribed exclusively to the change in the mode of respiration of the bacterium because the effects of the metallate on other metabolic processes cannot be ruled out completely, the results were consistent with the data suggesting that enzyme activities found in anaerobic respiratory chains may be more susceptible to redox and specific inhibitory effects by vanadium ions than those found in aerobic respiratory chains (Table 3).

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