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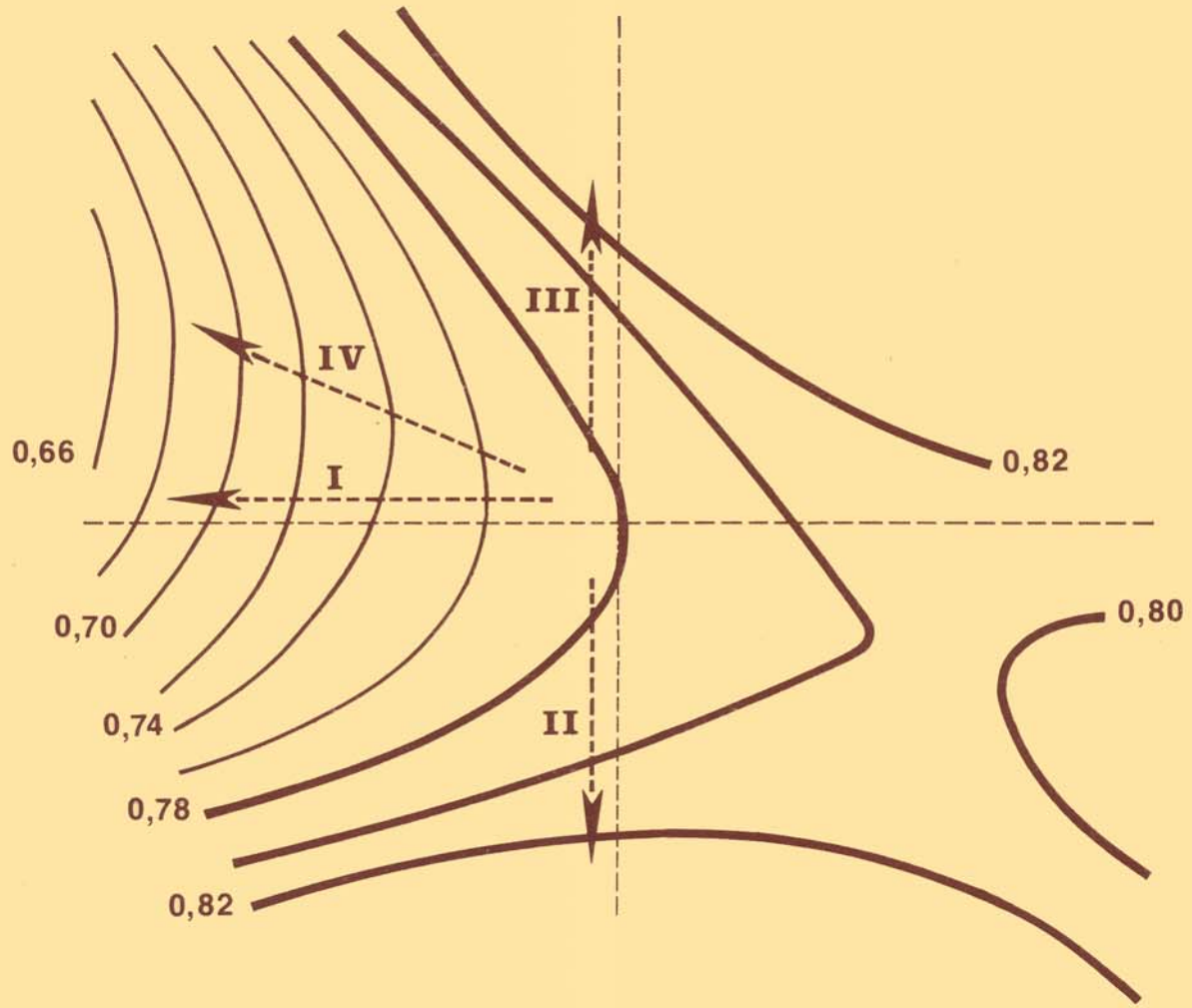

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RELATIONSHIP BETWEEN THE NITRATE AND OXYGEN RESPIRATORY SYSTEMS IN MEMBRANE VESICLES OF ESCHERICHIA COLI K-12. EFFECT OF 2-N-HEPTYL-4-HYDROXYQUINOLINE-N-OXIDE AND ULTRAVIOLET LIGHT

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ABSTRACT

Membrane vesicles prepared from anaerobic nitrate-grown *Escherichia coli* cells were shown to contain both oxygen and nitrate reducing systems. Ascorbate-reduced phenazine methosulfate and NADH-reduced menadione were used as artificial electron donors for the enzymatic reduction of nitrate. By using ascorbate phenazine methosulfate, it was shown that nitrate reductase is essentially not inhibited by molecular oxygen. Nitrate and oxygen compete for available reducing equivalents from NADH or formate. Electrons proceeding from oxidation of NADH and formate pass through common electron carriers. This favors the idea that electron transfer chains in *E. coli* are of the ramified type. 2-N-heptyl-4-Hydroxyquinoline-N-oxide (HQNO) inhibited electron transfer to oxygen and nitrate in at least one site. Arguments are given which support the idea that these sites are also targets for ultraviolet light. Surprisingly, ultraviolet light irradiation inhibited the benzyl viologen mediated reduction of nitrate in membranes, but not in purified nitrate reductase.

RELACION ENTRE LOS SISTEMAS RESPIRATORIOS DEL OXÍGENO Y EL NITRATO EN VESÍCULAS DE MEMBRANA DE ESCHERICHIA COLI *K-12. EFECTO DEL 2-N-HEPTIL-4-HIDROXIQUINOLINA-N-OXIDO Y DE LA LUZ ULTRAVIOLETA*

RESUMEN

Fueron obtenidas vesículas de membrana, preparadas a partir de células de *Escherichia coli* crecidas en anaerobiosis con nitrato, que contienen sistemas reductores del oxígeno y del nitrato. Estas vesículas son capaces de reducir el nitrato en presencia de dos donadores artificiales de electrones: Fenacina metasulfato reducido por ascorbato y menadiona reducido por Nicotinamida adenindinucleótido reducido (NADH). Con el primero de estos donadores, se demostró que la enzima nitrato reductasa no es inhibida por oxígeno molecular. El NO_3^- y el O_2 compiten por equivalentes reductores provenientes del NADH o del formiato. Los electrones producidos por la oxidación de estos donadores pasan a través de transportadores que son comunes a las cadenas respiratorias del O_2 y de NO_3^-. Estas observaciones son argumentos en favor del carácter ramificado de las cadenas respiratorias en *E. coli.* 2-N-heptil-4-hidroxiquinolina-N-óxido (HQNO) inhibió la transferencia de electrones hacia el O_2 y el NO_3^- en, al menos, un sitio de la cadena. Los argumentos presentados confirman la idea de que la luz ultravioleta actúa también sobre estos sitios. Sorpresivamente, la irradiación de las vesículas con luz ultravioleta inhibió la reducción del nitrato dependiente del benzil viológeno pero esa irradiación no afecta a la nitrato reductasa purificada.

INTRODUCTION

Pichinoty observed some years ago that nitrate reduction in anaerobically grown *E. coli* cells was inhibited by molecular oxygen.[15] Two hypotheses were presented to explain this observation. The first one suggests a direct inhibition of nitrate reductase, while the second one implies a competition for respiratory chain electrons between oxygen and nitrate.[15] Until now, the problem was not resolved, since all artificial electron donors used for nitrate reduction are too auto oxidizable to check the possible inhibitory action of oxygen upon this enzyme.

On the other hand, a competition between oxygen and nitrate for available reducing equivalents would imply the existence, in these anaerobically grown cells, of a specific and constitutive oxygen respiratory complex or, at least, the presence of an auto oxidizable electron carrier. This problem has not yet received a satisfactory answer.

In the present work, ascorbate-reduced phenazine methosulfate was used as a donor for nitrate reduction. Its auto oxidizability was low enough to study the direct effect of oxygen upon nitrate reductase. We also characterized a specific oxidase system in anaerobically grown cells of *E. coli* and its relationship with the nitrate reducing complex.

Generally it is thought that formate is specific for nitrate reduction and NADH for oxygen respiration.[8] Earlier workers[9] suggested, without demonstration, that both donor systems can act simultaneously. However, little is known about the relationship between these two electron donor systems. Our report deals with this problem, since the membrane vesicles prepared in this work could reduce both nitrate and oxygen in the presence of either NADH or formate. A more detailed characterization of this complex electron pathway was obtained through the use of respiratory inhibitors, such as HQNO (2-N-Heptyl-4-Hydroxyquinoline-N-Oxide) and ultraviolet light.

MATERIAL AND METHODS

Cultures and Growth Conditions

E. coli K_{12} Hfr P_4X, met^-, 303 in Puig's collection was anaerobically grown at 37° C in the following medium: 0.4 g

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NH_4Cl, 0.4_g $MgSO_4$ $7H_2O$, 1_g bactopeptone, 0.5_g meat extract, 0.5_g yeast extract, 2_g glucose, and 1_g KNO_3 in one liter of phosphate buffer 0.06M pH 8.0. Sodium azide was added to the growth medium at a concentration of 0.1 mM.

The cells were collected one hour before the beginning of the stationary phase, and then washed three times with Tris-HCl buffer 0.01M, pH 7.3, in order to eliminate nitrite and azide.

Preparation of Membrane Vesicles

Vesicles were prepared essentially according to the method of Corao *et al.*[1] The most important modification was the reduction of resuspension volume of the cells to one tenth of its original value.

Determination of Enzymatic Activity

Nitrate reductase activity was determined through nitrite production according to the method of Snell *et al.*[22] when NADH, formate, menadione-reduced NADH or ascorbate phenazine methosulfate were utilized. Nitrate reductase activity was also determined through hydrogen consumption in the presence of hydrogenase and benzyl viologen.[15].

Oxidase activity was determined by measuring the oxygen uptake in a Gilson oxygraph with a recorder attachment. All experiments were performed at 25° C. At this temperature and at a pressure of 760 mm Hg, dissolved oxygen concentration is considered to be 240 μM.

Formate dehydrogenase activity was determined manometrically in the presence of phenazine methosulfate as an electron acceptor.[17]

NADH-dehydrogenase activity was measured in the presence of ferricyanide.[6] Superoxide dismutase was a gift from Hatchikian.[8] Following the method of this author, xantine dehydrogenase was also used.

Other Assay Techniques

Proteins, mucleic acids, and keto-deoxyoctonic acid were determined according to the methods of Lowry *et al.*,[12] Schneider,[20] and Wassbach *et al.*,[23] respectively.

RESULTS

Characterization of Membrane Vesicles

Twenty two and one half % of the proteins, 0.5 % of the nucleic acids of the whole cells, and 8 % of the cell wall (measured as ketodeoxyoctonic acid content) remained in the membrane vesicles. The vesicular preparation also lost the cytochrome c_{552}, which means that periplasmic proteins had been eliminated.[5] The vesicles appeared as [illegible]y, motionless structures when observed with a phase-contrast microscope, and as closed little bags when observed with an electron microscope. Their size varied from 0.3 to 4 μm. All activities related to glycolysis or nitrite reduction had been lost.

These membrane vesicles allowed the reduction of both oxygen and nitrate by formate and NADH (Table I). Both of these systems were inhibited by HQNO. Formate, as expected, was the best electron donor for nitrate reduction[18] but also for oxygen respiration. The high level of NADH-nitrate reductase activity was in contrast with most previous publications, but in agreement with a report of Lester and De Moss.[11] CO inhibited oxygen reduction in the presence of NADH or formate. These results support the presence of both oxygen and nitrate reducing electron pathways in nitrate anaerobically grown *E. coli* cells. This was in agreement with a previous analysis of cytochromes b of these bacteria.[19]

Phenazine-methosulfate as an Electron Donor

Contrary to what was previously reported in cell free extracts,[18] ascorbate was not an electron donor for nitrate reduction in our membrane vesicles. However, it was found that the addition of phenazine methosulfate as a mediator between ascorbate and membrane enzyme produced nitrate reduction. The reaction did not take place if membrane, phenazine methosulfate, or ascorbate, was omitted, or if the vesicles had been previously heated at 100° C for 30 minutes. The rate of nitrate reduction was a linear function of the membrane concentration. The optimum pH was between 6.5 and 7.5. The optimal concentrations were 1.10^{-4}M and 2.10^{-2}M for phenazine methosulfate and ascorbate respectively.

The use of this electron donor did not modify the Km value of nitrate reductase.[4] A pseudo Km value of 1.3×10^{-5}M was obtained with respect to phenazine methosulfate. The reduction of nitrate in the presence of ascorbate-phenazine methosulfate was inhibited 80 % by azide (1 mM), 98 % by cyanide (1 mM), and 50 % by HQNO (0.13 mM), but arsenate (1 mM) had no effect on this activity.

Nitrate reductase, purified according to Forget[4] was not longer able to reduce nitrate in the presence of ascorbate-phenazine methosulfate. This indicates that the electrons are not transferred directly to the terminal enzyme, but to some intermediate electron carrier.

TABLE I

MEMBRANE VESICULAR ACTIVITIES RELATED WITH THE REDUCTION OF NITRATE AND OXYGEN

Donor	*Acceptor*	*Activity μM of reduced acceptor mg of protein per minute*
Formate	PMS	1.1
Formate	NO_3^-	0.18
Formate	O_2	0.10
NADH	Ferricyanide	1.8
NADH	NO_3^-	0.06
NADH	O_2	0.04
BV	NO_3^-	8.7
Menadione	NO_3^-	0.60

Membrane vesicles (1 mg of protein/ml) are suspended in a 0.06 M phosphate buffer, pH 7.3. Nitrate is 10^{-2}M, oxygen 240 μM. Phenazine Methosulfate (PMS) and ferricyanide are 10^{-3}M. NADH and formate are 5×10^{-3}M and 10^{-3}M respectively. Benzyl viologen is maintained reduced by hydrogenase and hydrogen,[16] while menadione is enzymatically reduced by NADH in the presence of rotenone (the absence of rotenone increases the activity by 10 %). Temperature 25° C.

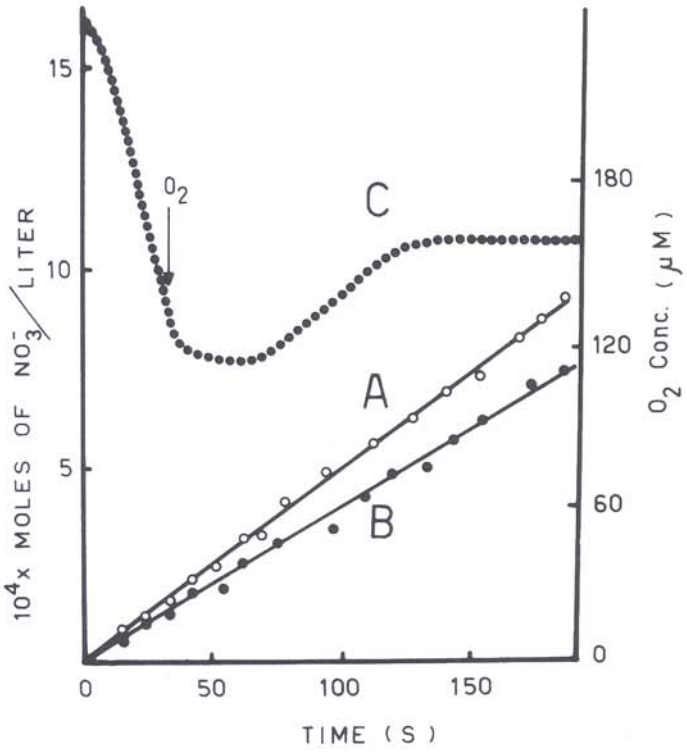

Figure 1. *Nitrate Reduction by Vesicular Membranes in the Presence of Ascorbate Phenazine Methosulfate Under Controlled Oxygen Content.* Phenazine methosulfate was 10^{-4}M, ascorbate 2.10^{-2}M. The determination of oxygen concentration was by polarography and by colorimetry for nitrate, as indicated in «Materials and Methods». Curve A is a control of nitrate reduction in anaerobiosis. Curve B is the production of nitrite under aerobic conditions given by Curve C, where oxygen concentration was maintained between 120 and 150 μM by air bubbling.

Surprisingly, nitrate reduction was only slightly affected by air bubbling. Vigorous pure oxygen bubbling only decreased the nitrate reduction by 10-20 %. Since reduced phenazine methosulfate is autooxidizable, and the oxygen is poorly soluble in water, care was taken to do the determinations under conditions where oxygen dissolution was not the limiting step.

In a manometric experiment, in a pure oxygen atmosphere, the mixture of 3.10^{-3}M ascorbate and $1.3\ 10^{-5}$M phenazine methosulfate was found to be the adequate. In effect, a mixture with a concentration seven times higher gave a four-fold increase in oxygen consumption. Under such conditions, nitrate reduction proceeded at 85 % of the rate measured in the absence of oxygen.

Results shown in Figure 1 confirm that a high oxygen concentration (120-150 μM) produced only a slight inhibition of nitrate reduction. There remained the possibility of a chemical reduction of nitrate by the superoxide ion produced by the reaction of molecular oxygen with reduced phenazine methosulfate.[14] Neither the addition of pure superoxide dismutase (40 μg/ml), nor the artificial production of this superoxide ion our system (xanthyne dehydrogenase, 50 μg/ml with its subsubstrate 10^{-5}M), affected the reduction of nitrate (data not shown).

These results prove that nitrate reductase can work in the presence of a high oxygen concentration and that aerobic reduction of nitrate is not a chemical artifact.

Relationship Between Oxygen and Nitrate Reduction Systems

When nitrate and oxygen were present simultaneously, the reduction of each of these electron acceptors was slower than when the other acceptor was not present (Table II). Under these conditions, nitrate inhibited oxygen consumption more (85-90 % inhibition in the presence of formate, 70 % in the presence of NADH) than oxygen affected nitrate reduction.

The experiment presented in Figure 2 shows that oxidase activity was not directly affected by nitrate. In effect, azide 10^{-4}M, which decreased nitrate reductase activity to 10 % of its original value, released almost completely oxidase inhibition

TABLE II

Reduction of Nitrate and Oxygen by NADH or Formate Effect of CO

	Electron Acceptor	NO_3^-	$NO_3^- + O_2$		O_2	
Electron Donor	*Activity*	*Nitrate Reductase 1*	*Nitrate Reductase 2*	Oxidase *3*	*Oxidase −CO 4*	*+CO 5*
Formate		400	130	17	148	43
NADH		85	59	17	60	20
Formate and NADH		340	195	37	193	38

All systems contain 0.75 mg of protein/ml. Formate and NADH were 5.10^{-3}M, $NO_3^-\ 10^{-2}$M, oxygen 240 μM. CO was bubbled until a level of 100μM of dissolved oxygen was reached. When CO was absent, the oxygen level was lowered to 100 μM by argon bubbling. The temperature was 25° C. Oxygen uptake was measured by polarography. Simultaneously 20 μl aliquotes were withdrawn from the measuring cell to determine the NO_2^- content. Anaerobiosis was obtained by argon bubbling. Results are expressed in reductor microequivalents to compare both oxygen and nitrate reductions on the same basis. One reductor microequivalent represents 0.5 μM of reduced oxygen or 1 μM of produced nitrite.

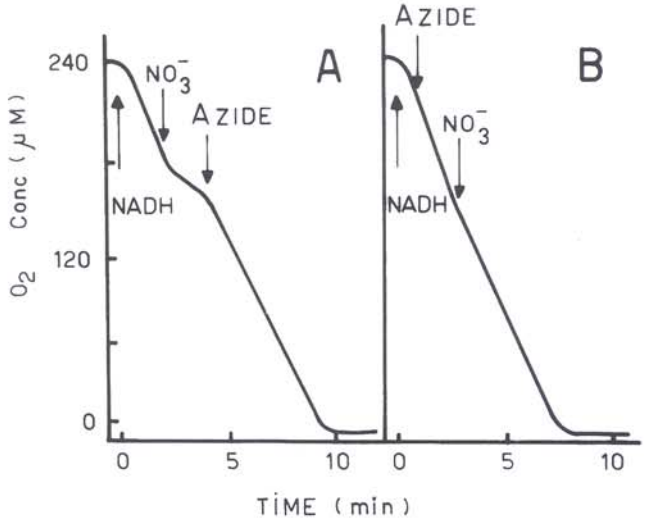

Figure 2. *Effect of Azide on the Simultaneous Reductions of Oxigen and Nitrate by Vesicular Membranes.* The systems (2.0 ml) contain 0.8 mg/ml of proteins, 5×10^{-3}M of NADH and 240 μM of oxygen. When added, azide is 10^{-4}M and NO_3^- is 10^{-2}M. Temperature 25° C. Curve A: nitrate is added before azide. Curve B: Azide is added after nitrate.

by nitrate (Figure 2A). In the symmetrical experiment, azide prevented the inhibitory effect of nitrate (Figure 2B). Similar results were obtained with formate.

Figure 3 shows that the decrease of oxygen uptake depends on nitrate concentration in an hyperbolic way. Since nitrite, which cannot be reduced by the membrane vesicles, does not produce any oxygen uptake inhibition, the inhibition must depend on the functionning of nitrate reductase.

Table II (columns 2 and 3) shows that, in the presence of NADH as donor, the sum of the oxygen and nitrate reduction rates was nearly equal to the individual rates of either reduced oxygen (column 4) or reduced nitrate (column 1). This indicates that electrons provided by NADH dehydrogenase were shared between the nitrate and oxygen reduction systems. Similar conclusion can be reached in the case of formate, assuming an oxygen inhibition of formate dehydrogenase. Enoch and Lester[3] have recently shown that the purified formate dehydrogenase is sensitive to molecular oxygen. We have verified that this situation also exists for the membrane bound enzyme (data not shown). All these results are consistent with the fact that nitrate and oxygen compete for electron equivalents from NADH or formate.

Relationship Between NADH and Formate Dehydrogenase Systems

Columns 2,3 and 4 of Table II show the additivity of electron donating powers of formate and NADH. This phenomenon is attributed to a partial inhibition of formate dehydrogenase by oxygen, by limiting the electron-transfer rate on the dehydrogenase side of the chain. In the absence of oxygen, formate dehydrogenase is fully active and the additivity phenomenon does not occur (column 1, Table II). Under such conditions, formate alone, as well as mixtures of NADH and formate, give the same theoretical maximum velocity (Figure 4). This indicates that both formate and NADH give electrons to a common rate-limiting part of the respiratory chain.

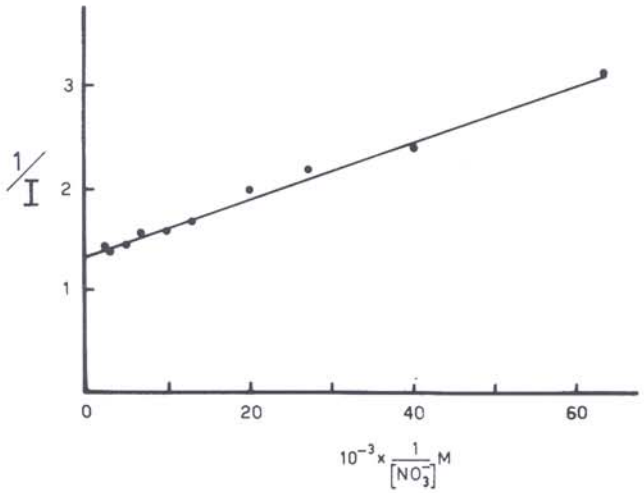

Figure 3. *Inhibition of NADH-Oxidase Activity as a Function of the Nitrate Concentration. Lineweaver-Burk Plot.* Conditions are the same as in Figure 2. I is the inhibition of oxygen consumption by nitrate, expressed as the ratio of inhibited activity and maximum activity. Pseudo K_M is 2×10^{-5}M.

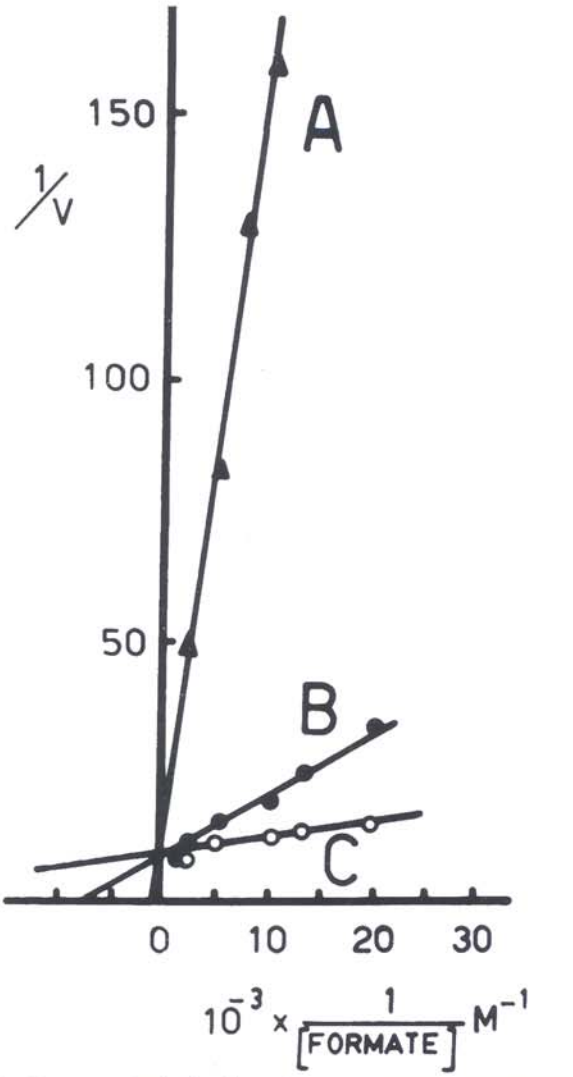

Figure 4. *Lineweaver-Burk Plot of Vesicular Nitrate Reductase Activity Versus Formate Concentration in the Presence of Increasing NADH Concentration.* Experimental conditions were: temperature 25° C; anaerobiosis (argon bubbling); nitrate 10^{-2}M. Activity was determined by nitrite production. Curve A is with formate only. Curve B is with a constant NADH concentration of 10^{-3}M. Curve C is with 2.10^{-3}M NADH.

When oxidase activity was inhibited by CO bubbling (Column 5 Table II), NADH did not increase the oxygen consumption above that due to formate. This excludes the existence of a predominant NADH specific oxidase pathway and argues in favour of the existence of a central electron transport pool, receiving from both formate and NADH and donating to both nitrate and oxygen.

Inhibition of Nitrate and Oxygen Respiratory Systems by HQNO and Ultraviolet Light

HQNO inhibited the nitrate reductase activity in different ways, depending on the electron donor. When formate or NADH were the electron donor, 50 % inhibition was obtained with 30 μM of HQNO; the same result was achieved with 130 μM of HQNO in the presence of ascorbate phenazine methosulfate or NADH-reduced menadione. The maximun inhibition (obtained with 400 μM of HQNO) was 80 % in the

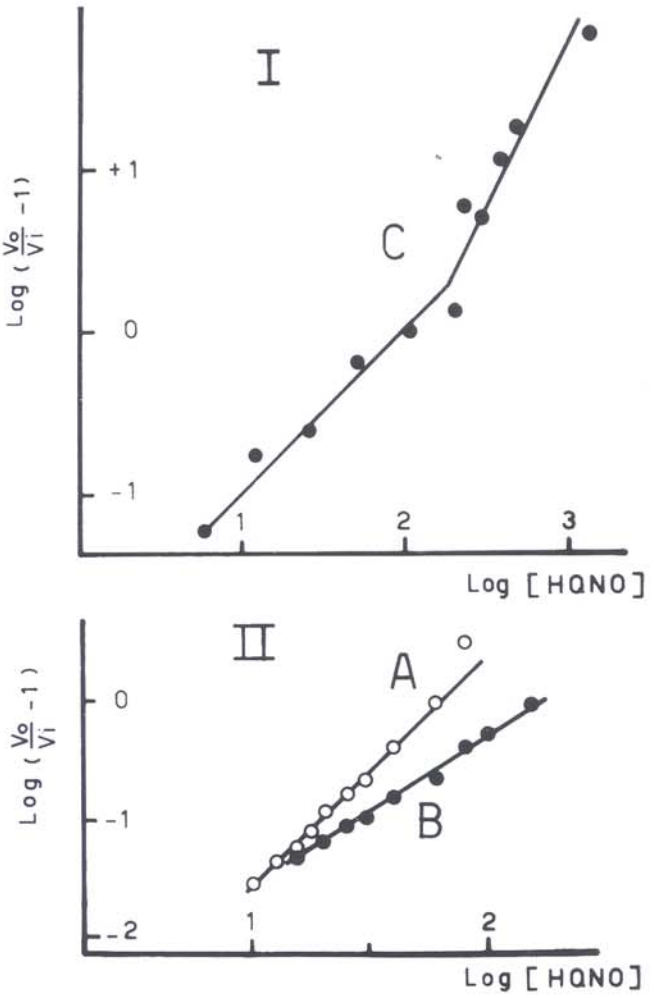

Figure 5. *Hill Plots for the Inhibition of the Activities APM-Nitrate Reductase, Formate-Nitrate Reductase and Formate-Oxidase by HQNO.* The membrane vesicles (0.8 mg of protein/mg) were suspended in 0.06M phosfate buffer (pH 7.3). Final volume 2 ml APM: Ascorbate 2.10^{-2}M and phenazine methosulfate 10^{-4}M. Formate 5.10^{-3}M, nitrate 10^{-2}M, oxygen 240 µM. HQNO was added three minutes before the electron donor. V_O is the activity in the absence of the inhibitor and V_i is the activity in the presence of increasing amount of HQNO. V_o and V_i were corrected for the residual enzymatic activities that could not be inhibited by HQNO. Curves A and B represent the inhibition of nitrate reductase activity in the presence of formate and ascorbate-reduced phenazine methosulfate (APM) respectively. Curve C represents the inhibiton of the oxidase activity in the presence of formate.

presence of NADH, formate, or NADH-reduced menadione. However, the ascorbate phenazine methosulfate-nitrate reductase activity cannot be inhibited more than 60 %. These results indicate the presence of more than one inhibition site.

An analysis of inhibition as a function of HQNO concentration according to Muijsers *et al.*[13] is presented in Figure 5. The fact that all the slopes of the lines gave values greater than 1 supports the idea that HQNO reacts at more than one site, in agreement with an observation of Cox *et al.*[2]

Table III shows the effect of ultraviolet light irradiation on the nitrate and oxidase reduction systems. Irradiation was performed under conditions such that FMN (Flavin mononuleotide) was only slowly photolysed (t 1/2 = 2 hours). The rapid inhibition of membrane bound nitrate reductase activity in the presence of benzyl viologen was surprising since purified nitrate reductase (Forget's type) was not affected by ultraviolet light (Table III). A likely explanation is that benzyl viologen donates electrons directly to solubilized nitrate reductase and both directly and through a carrier, sensitive to ultraviolet irradiation, to the membrane enzyme. The reduced benzyl viologen acceptor site located on nitrate reductase would be less efficient since the inhibition of this activity can reach 60 %.

Almost all measured activities were affected by ultraviolet light irradiation (Table III). Phenazine methosulfate-nitrate reductase activity can be lost completely upon sufficient irradiation through a first order kinetics, characterized by a t 1/2 of 60 minutes. NADH and formate-nitrate reductase systems can also be completely inhibited, whereas NADH-oxidase cannot be inhibited by more than 70 %. The inhibition kinetics were of first order until 20-25 % of residual activity was reached with similar half-time values (Table III). These results indicate the presence of various ultraviolet light sensible sites.

When membrane vesicles were simultaneously treated with HQNO and ultraviolet light, the inhibitory effects were less than additive (Table IV). This result seems to indicate that both inhibitors actuate on the same electron-transfer steps. If the electron pathway is considered analogous to a hydrodynamic system, it is possible to calculate the increment of inhibition due to a second inhibitor when both inhibitors actuate on the same point. Such calculations conrrespond fairly well to our experimental results (Table IV). The fact that at least one site of the chain is sensitive to both HQNO and ultraviolet light is in agreement with other authors.[2].

TABLE III

INHIBITORY EFFECT OF ULTRAVIOLET IRRADIATION

System	*Maximum Inhibition (%)*	$t_{1/2}$ *(minutes)*
1. Formate – Nitrate Reductase	95	25
2. NADH – Nitrate Reductase	95	30
3. Ascorbate Phenazine methosulfate (NADH reduced)	90	60
4. Menadione – Nitrate Reductase	80	38
5. Benzyl Viologen – Nitrate Reductase (Membrane)	60	28
6. BV – Purified Nitrate Reductase	0	No inhibition
7. NADH – Ferricyanide	50	240
8. Formate – Phenazine methosulfate	60	40
9. NADH – Oxidase	70	28

The membrane vesicles (0.8 mg of protein/ml) are suspended in a phosphate buffer 0.06 M. Formate and NADH are 5.10^{-3}M. Ascorbate phenazine methosulfate is 2.10^{-2}M in ascorbate and 10^{-4}M in phenazine methosulfate. Menadione (10^{-4}M) is enzymatically reduced in the presence of NADH. Nitrate is 2.10^{-2}M. Aliquotes are withdrawn at different times and analyzed for nitrite (systems 1-6). The activities of systems 7-9 are measured as indicated in Table I. Irradiation is performed with a Mineralight UV-lamp placed at 15 mm above the vesicular suspension. The suspension (3 mm thick) is maintamed at 4° C under constant stirring. The reaction vessel is kept closed by a 4 mm filter which lets light pass between 320 and 400 nm. $t_{1/2}$ is the time which enables the loss of half of the activity which can be inhibited.

TABLE IV

SIMULTANEOUS EFFECT OF HQNO AND ULTRAVIOLET IRRADIATION

	Formate-Oxidase system			*NADH-Oxidase system*		
Inhibitor	*Activity*	*Inhibition %*	*Predicted Inhibition %*	*Activity*	*Inhibition %*	*Predicted Inhibition %*
None	104.0	0	–	43.0	0	–
HQNO	35.5	66	–	10.0	77	–
Ultraviolet Light (15′ min)	59.6	43	–	31.0	28	–
Ultraviolet Light (35′ min)	40.3	61	–	27.5	36	–
HQNO + Ultraviolet Light (15′ min)	18.3	82	81	7.5	83	83
HQNO + Ultraviolet Light (35′ min)	8.2	92	87	7.0	84	85

Formate and NAD were 5×10^{-3}M and HQNO was 70 μM. Experimental conditions were the same as in Table 3.

The predicted inhibition (considering that both inhibitors have the same target) is calculated as follows: inhibition due to inhibitor I + (inhibition due to inhibitor II, multiplied by the percent of the residual native target). Activies are expressed in μM of O_2 reduced/minute.

DISCUSSION

Ascorbate-reduced phenazine methosulfate and NADH-reduced menadione are defined in this work as new electron donors for nitrate reduction. The first of these artificial donors is of particular importance since it enabled us to demonstrate that *E. coli* nitrate reductase is not inhibited by molecular oxygen. The little decrease of nitrate reduction observed in the presence of oxygen may be attributed to a competition for reduced phenazine methosulfate between the electron transfer chain and oxygen. This demonstration finally resolves the problem outlined by the observations that anaerobic nitrate induced *E. coli* cells were unable to reduce nitrate in the presence of oxygen.[15, 21] Pichinoty's hypothesis,[15] based on a competition between oxygen uptake and nitrate reduction, is supported by this work, which shows that a specific and constitutive oxygen electron-transfer chain does exist in the anaerobically grown *E. coli* cells. It was also shown that nitrate and oxygen compete for available reducing equivalents. However, the relative electron transferring capacities which we have observed for these two acceptor systems do no support an inhibitory effect of O_2 on nitrate reduction, but rather a nitrate inhibition of oxygen consumption. This contradiction with previous reports[7, 15] does not seem to be due to the use of membrane vesicles instead of intact cells since oxygen consumption was also inhibited by nitrate in unbroken cells in the presence of glucose. It might rather be attributed to the presence of azide in our culture medium. This inhibitor is known to increment the nitrate reductase synthesis (Chippaux M., Thèse d'Etat, Marseille, 1973).

Both NADH and formate were shown to give electrons for either oxygen or nitrate reduction through some common electron carriers. Therefore the electron transfer pathway of anaerobically nitrate grown *E. coli* cells can be represented as the assembly of several electron-donor and electron-acceptor specific complexes around a pool of common carriers, like quinones. Analogous results have been obtained with *Klebsiella aerogenes*[10] and *E. coli*.[19]

Some experiments presented in this work suggest that both ultraviolet light (360 nm) and HQNO can inhibit the electron transfer chain in more than one point. Moreover at least one site would be common to both inhibitors. By analogy with mitochondria it is considered that the principal target of ultraviolet light is the central pool of quinone molecules. An inhibitory site of HQNO is also reported in this part of the chain.[18] Support to the multiplicity of inhibition sites by 360 nm light is given by the effect of irradiation upon benzyl viologen mediated nitrate reduction. While vesicular nitrate reductase activity was significantly decreased by this treatment, purified nitrate reductase was not affected. It indicates that reduced benzyl viologen does not donate electrons to the same site in the purified and membranar nitrate reductase. This could explain the slight difference in Km values observed by Forget.[4] While the purified nitrate reductase used in his experiments did not contain any trace of heme,[4] membranous enzyme is known to be closely associated to a cytochrome b.[3, 19] A likely explanation is that the ultraviolet light sensitive site is located between the cytochrome b and the nitrate reductase.

The presence of a HQNO inhibition site between cytochrome b and the terminal reductase is supported by cytochrome b reoxidation experiments.[19] In effect, it was observed that the reoxidation of the cytochromes b specific of nitrate reductase and oxidase complexes is greatly affected by HQNO. In the partially dissociated electron transfer chain (unpublished data), the nitrate and oxygen reduction complexes being separated, essentially the same results are obtained.

At this point it is important to recall that our conclusion is in agreement with that of Cox *et al.*[2] on a different experimental basis. These authors observed that HQNO inhibits NADH-oxidase electron transfer chain at two sites and that these sites are likely to correspond to iron-quinone pools.

This introduces the possibility that a hydrogen redox carrier be present between cytochrome b (specific of the reductase complex) and the terminal reductase. A recent study on mitochondrial quinones gives some support to this hypothesis.[24]

Although further work is obviously required to characterize unambiguously such a quinone in the reductase complex, the available data may suggest the existence of another redox proton translocating segment in *E. coli* respiratory chain.

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