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Sites and Specificity of the Reaction of Bipyridylium Compounds with Anaerobic Respiratory Enzymes of *Escherichia coli*

EFFECTS OF PERMEABILITY BARRIERS IMPOSED BY THE CYTOPLASMIC MEMBRANE

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The ability of the oxidized and singly reduced species of several bipyridylium cations to cross the cytoplasmic membrane of *Escherichia coli* was studied to locate the sites of reaction of the dyes with anaerobic respiratory enzymes. Benzyl Viologen radical crossed the membrane rapidly, whereas the oxidized species did not. The oxidized or radical species of Methyl Viologen, Morfamquat or Diquat did not rapidly cross the membrane. It was also shown that the dithionite anion does not cross the cytoplasmic membrane of *E. coli*. Diquat radical donates electrons to the nitrate reductase pathway at the periplasmic aspect of the membrane, whereas Benzyl Viologen radical reacted directly with nitrate reductase itself (EC 1.7.99.4) at the cytoplasmic aspect of the membrane. Thus the pathway of electron transfer in the nitrate reductase pathway is transmembranous. Formate hydrogenlyase (EC 1.2.1.2) and an uncharacterized nitrite reductase activity react with bipyridylium dyes at the periplasmic aspect of the membrane. Fumarate reductase (succinate dehydrogenase; EC 1.3.99.1) reacts with bipyridylium radicals, and formate dehydrogenase (cytochrome) (EC 1.2.2.1) with ferricyanide, at the cytoplasmic aspect of the membrane. The differing charge and membrane permeation of oxidized and radical species of bipyridylium dyes greatly complicate their use as potentiometric mediators in suspensions of cells or membrane vesicles.

Bipyridylium compounds* such as the oxidized or radical species of Methyl Viologen and Benzyl Viologen have been widely used as low-potential electron acceptors or donors in biological systems (Clark, 1972). Methyl Viologen ('Paraquat') and Diquat are also used as herbicides (Dodge, 1971). Gage (1968) showed that oxidized Methyl Viologen and Diquat did not cross the inner membrane of rat liver mitochondria. Hauska *et al.* (1974) and Saha *et al.* (1971) regarded Methyl Viologen as a hydrophilic compound unable to cross the membranes of chloroplasts, and a similar view was taken by Anderson *et al.* (1976) of the inability of Methyl Viologen to cross artificial lipid membranes. However, the single positive charge of Methyl Viologen radical is delocalized, and that of Benzyl Viologen radical is presumably even more so, in view of the difference in oxidoreduction potentials (Table 1). Organic cations with delocalized charges can in some instances cross biological and artificial lipid membranes, particularly if the molecule also contains hydrophobic groups. It therefore seemed likely that some bipyridylium radicals would be unable to cross membranes. We have confirmed this prediction, and used it in demonstrating that the anaerobic respiratory pathway of nitrate reduction involves electron transfer across the cytoplasmic membrane. We have also used bipyridylium compounds and other oxidoreduction reagents to determine the intracellular location of several enzyme activities that are characteristic of various modes of anaerobic growth. A preliminary report of part of this work has already been published (Jones *et al.*, 1976).

* Bipyridylium compounds can exist as the fully oxidized species, the singly reduced radical species, or fully reduced species. There is not a suitable general terminology for this class of compounds. Not all have violet-coloured radicals, hence 'Viologen' is inappropriate. The presence of charged substituents would confuse a description of oxidoreduction state based on net molecular charge. We will use commerical names for each compound described here, and refer to the fully oxidized species as 'oxidized', the singly reduced species as the radical, and the fully reduced species as such. All four compounds described here are bivalent cations when oxidized. Their radicals are univalent cations. Table 1 summarizes some of their properties.

Table 1. *Properties of the four bipyridylium compounds used in the present study*

The values for redox potential (E_0) and millimolar extinction coefficient (ε_{mM}) refer to interconversion of the fully oxidized species to the radical. References: [a]Homer *et al.* (1960); [b]Baldwin (1969); [c]Thorneley (1974); [d]Lester & De Moss (1971); [e]R. W. Jones, unpublished work; [f]J. A. Farrington, personal communication.

Trivial name of cation	Generic name	E_0 (mV)	$\lambda_{max.}$ (nm)	ε_{mM} ($l \cdot mmol^{-1} \cdot cm^{-1}$)
Benzyl Viologen	1,1′-Dibenzyl-4,4′-bipyridylium (dichloride)	−350[a]	600[d]	7.4[d]
Diquat	1,1′-Ethylene-2,2′-bipyridylium (dibromide)	−349[b]	460[e]	2.7[e]
Methyl Viologen	1,1′-Dimethyl-4,4′-bipyridylium (dichloride)	−446[a]	600[c]	13[c]
Morfamquat	1,1′-Bis(dimethylmorpholinocarbonylmethyl)-4,4′-bipyridylium (dichloride)	−374[f]	600[e]	9.0[e]

Materials and Methods

Organisms

Escherichia coli strains A1002 and A1004a (Haddock, 1973) were generously given by Dr. H. U. Schairer (Max-Planck-Institut für Biologie, 73-Tubingen, West Germany). Strain A1004a, unlike its parent A1002, is unable to synthesize 5-aminolaevulinic acid and is therefore haem-deficient unless the growth medium is suitably supplemented.

Growth of parent strain A1002

All cultures were made anaerobically at 37°C. Stock cultures of strains were kept as 4ml suspensions in 15% (v/v) glycerol at −22°C. Preliminary overnight cultures were made by inoculating 100ml of the medium (see below) with 0.2ml of stock culture. These preliminary overnight cultures were stored at 4°C and used to provide 2.0ml portions for starting large-scale cultures of 500ml on the subsequent night. The large-scale cultures were harvested after growth for 12–16h. The following media were used. Preliminary overnight culture: nutrient broth (10g of tryptone, 5g of yeast extract, 7.5g of KCl, 12g of K_2HPO_4, 3g of KH_2PO_4, in 1 litre) supplemented with L-isoleucine, L-valine and L-methionine (20μg/ml in each case). Large-scale culture: glycerol (0.5%, w/v), casamino acids (0.1%, w/v), 10mM-$MgCl_2$, 1μM-$(NH_4)_6Mo_7O_{24}$, 1μM-K_2SeO_3, L-valine (20μg/ml), L-isoleucine (20μg/ml) and L-methionine (20μg/ml) in 500ml of the medium of Cohen & Rickenberg (1956) containing one of (*a*) KNO_3 (1%, w/v), (*b*) disodium fumarate (1%, w/v) or (*c*) disodium fumarate (1%, w/v) with potassium formate (0.5%. w/v), which yielded cells induced respectively for (*a*) nitrate reductase (EC 1.7.99.4), (*b*) fumarate reductase (succinate dehydrogenase; EC 1.3.99.1), or (*c*) fumarate reductase and formate hydrogenlyase (EC 1.2.1.2) activities.

Growth of strain A1004a

Growth of satisfactory yields of this strain is complicated by the tendency of revertants to appear, and by the low growth yield. The following procedure and media were used. First a preliminary overnight culture was grown as for strain A1002, except that the medium also contained glucose (0.5%, w/v) and 5-aminolaevulinic acid (4μg/ml). The culture was stored at 4°C for a few hours, then used to start a second overnight culture of 4×250ml in a medium composed of glucose (0.5%, w/v), casamino acids (0.1%, w/v), 10mM-$MgCl_2$, L-valine (20μg/ml), L-isoleucine (20μg ml), L-methionine (20μg/ml), 5-aminolaevulinic acid (0.04μg/ml), in Cohen & Rickenberg (1956) medium. Culture was in fully filled flasks placed inside an evacuated vacuum desiccator, without stirring or agitation. After 16h, most of the supernatant was carefully decanted, leaving a concentrated suspension of cells in the flasks, 2.0ml of which was used, after storage at 4°C for a few hours, to start a third overnight culture in 500ml of glucose (0.5%, w/v), casamino acids (0.1%, w/v), 10mM-$MgCl_2$, valine (20μg/ml), isoleucine (20μg/ml), methionine (20μg/ml), 1μM-$(NH_4)_6Mo_7O_{24}$, 1μM-K_2SeO_3 and KNO_3 (1%, w/v) in Cohen & Rickenberg (1956) medium. Culture was again in filled flasks in an evacuated vacuum desiccator.

Harvesting of cells and preparation of spheroplasts

Cells were harvested from cultures after about 16h of growth by centrifugation at 7000*g* for 15min at 0–4°C in the 6×750ml head of a MSE Mistral 6L centrifuge. Cells were washed thrice by resuspending them in their original culture volume in 50mM-KH_2PO_4/Na_2HPO_4, pH6.8, and centrifuging as before. The cells were resuspended in this buffer to give a cell protein concentration of approx. 10mg/ml, and stored at 0–4°C before use within 3–6h. Spheroplasts were prepared from harvested and washed cells as described previously (Garland *et al.*, 1975).

Assay of enzyme activities

Nitrate reductase activity was assayed spectrophotometrically at 600nm by using Benzyl Viologen, Methyl Viologen or Morfamquat radicals as electron donors, or at 460nm by using Diquat radical. The appropriate extinction coefficients are listed in

Table 1. Because the radicals are rapidly oxidized by oxygen, it was necessary to use an anaerobic technique. The following simple method was devised (it is free of the mechanical complications of bubbling and frothing usually associated with gassing techniques): A 1cm-light-path cuvette (type-21 stoppered cuvette from Starna Ltd., London E13 0PA, U.K.) equipped with a wide and thick Teflon stopper was used. The centre of the stopper had previously been drilled with a 0.75mm-diameter hole, giving a loose fit for a micro-syringe needle. To set up an anaerobic assay, a few anti-bumping granules were placed in the cuvette, to which was then added about 0.1ml of the cell or spheroplast suspension. The cuvette was then carefully filled just to the neck with N_2-saturated 20mM-Tris/HCl buffer, pH6.8 at 22°C, containing oxidized bipyridylium (0.5mM for Diquat, 0.3mM for Benzyl Viologen, Methyl Viologen and Morfamquat). The stopper was inserted, displacing excess of medium. The cell or spheroplast suspension at the bottom of the cuvette was then fully mixed with the medium above it by inverting the cuvette a few times to agitate the anti-bumping granules. During mixing the fine hole in the stopper was occluded with the operator's thumb, protected against contact with the toxic bipyridylium compounds by a plastic glove or piece of plastic film. Incomplete reduction of bipyridylium compounds to their radicals was achieved by adding, with a micro-syringe, small batches of freshly prepared 25mM-$Na_2S_2O_4$ in 10mM-NaOH until the absorption had risen to about 1.0. Nitrate reductase activity was measured from the rate at which the absorption of the radical fell after addition of 1M-KNO_3 (with a micro-syringe) to give a final concentration of 10mM-KNO_3. The working volume of the stoppered cuvette with granules was 3.5–3.6ml. This manual method of mixing interrupted the spectrophotometer recording for about 6s. In experiments where a faster response was needed, the cuvette was stirred magnetically with an 8mm-diameter magnetic stirrer (Nalgene star head type, from Techmate Ltd., Wheathampstead, Herts., U.K.) and a rotating magnet (Rank Bros., Bottisham, Cambridge, U.K.). The type of stoppered cuvette just described was chosen for its width of neck and ability to accept an 8mm-diameter plastic-coated magnetic disc.

Nitrite reductase and also fumarate reductase activity were assayed as for nitrate reductase with bipyridylium radicals as electron donors, except that 10mM-KNO_2 or disodium fumarate replaced KNO_3.

Formate dehydrogenase was assayed as an *N*-methylphenazonium methosulphate-dependent reduction of 2,6-dichlorophenol-indophenol in an anoxic cuvette. The N_2-saturated reaction mixture at 22°C contained 20mM-Tris/HCl buffer, pH6.8, 1mM-*N*-methylphenazonium methosulphate, about 0.1mg of cell protein/ml, and sufficient 2,6-dichlorophenol-indophenol to give an A_{600} of about 1.0. Formate dehydrogenase activity was measured as the rate at which the absorption fell after adding 2M-potassium formate to a final concentration of 20mM. In some experiments the membrane-impermeant *N*-methylphenazonium-3-sulphonate (Hauska, 1972) replaced *N*-methylphenazonium methosulphate. In other experiments, 2,6-dichlorophenolindophenol ($\varepsilon_{mM} = 21$ litre$^{-1}\cdot$mmol$^{-1}\cdot$cm^{-1} at 600nm; Lester & De Moss, 1971) was replaced by the more hydrophilic derivative, 2,6-dichlorophenol-indophenol-3-sulphonate ($\varepsilon_{mM} = 31$ litre$^{-1}\cdot$mmol$^{-1}\cdot$cm^{-1} at 650nm; R. W. Jones, unpublished work). Formate dehydrogenase was also measured by using ferricyanide as the electron acceptor in a medium containing 20mM-Tris/HCl buffer, pH6.8, 20mM-potassium formate and 1.0mM-potassium ferricyanide. The measuring wavelength was 420nm and $\varepsilon_{mM} = 1.0$ litre$^{-1}\cdot$mmol$^{-1}\cdot$cm^{-1} was assumed.

Nitrate reductase assays were also carried out by measuring the accumulation of nitrite in 2.0ml of a reaction mixture containing 20mM-Tris/HCl buffer, pH6.8 at 25°C, 20mM-Tris/ascorbate, pH6.8, 1mM-*N*-methylphenazonium ethosulphate or 1mM-*N*-methylphenazonium-3-sulphonate and 0.03–0.1mg of cell protein/ml. The reaction was carried out in open test tubes and initiated by the addition of KNO_3 to a final concentration of 10mM; after 2min a sample (0.5ml) was removed and added to 2.3ml of 0.1M-$ZnSO_4$ at 97–100°C. After cooling, 0.2ml of 0.5M-NaOH was added, and the mixture centrifuged with an MSE bench centrifuge to obtain a clear supernatant, which was then assayed for nitrite by the method of Nicholas & Nason (1957). Appropriate blanks were performed. Control experiments demonstrated that nitrite formation was dependent on both ascorbate and *N*-methylphenazonium ethosulphate.

Measurement of the uptake of oxidized bipyridylium compounds and their radicals by E. coli

The cells used were of parent strain A1002 grown anaerobically with glucose, de-repressed for nitrite reductase by inclusion of KNO_3 in the growth medium. To 3.6ml of 0.3mM oxidized bipyridylium compound in 20mM-Tris/HCl, pH6.8 at 22°C, was added 0.5ml (about 5mg of cell protein) of the harvested and washed cells. In some experiments 1mM-KNO_3 was also present. After 1min at room temperature (18–25°C) the cell suspension was divided into duplicate 1.5ml centrifuge tubes and centrifuged at maximal speed in an Eppendorf model 3200 centrifuge (Anderman, East Molesey, Surrey KT8 0QZ, U.K.). A portion (1.0ml) of supernatant was removed and assayed for bipyridylium compound by measuring the increase in A_{600} (or A_{460} for Diquat) on adding $Na_2S_2O_4$ (final concn. approx. 0.5mM at pH6.8). The pellet of centrifuged cells (volume <0.05ml) was similarly assayed after sus-

pension in 5ml of water and cell breakage by sonication with the $\frac{3}{8}$in (9.5mm) probe of an MSE 150W ultrasonic source at the maximum power for setting number 4. The cell suspension was surrounded by an ice bath and sonicated for four periods of 30s separated by periods of 15s. In some experiments the initial suspension of cells was placed in a spectrophotometric cuvette and the bipyridylium completely reduced to its radical by the addition of small portions of 10mM-$Na_2S_2O_4$ solution. After 1min the radical was oxidized either enzymically by adding 1mM-KNO_3 or non-enzymically with 1mM-H_2O_2. The cells were then separated from the suspension as above by centrifugation. No attempt was made to correct for contamination of the centrifuged cells by extracellular water.

Other methods

Protein was assayed by the method of Lowry *et al.* (1951) by using bovine serum albumin (fraction V; BDH Chemicals Ltd., Poole, Dorset, U.K.) as the standard.

Reagents

Lysozyme (EC 3.2.1.17) chloride, 5-aminolaevulinate hydrochloride, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and *N*-methylphenazonium methosulphate or ethosulphate were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames KT2 7BH, Surrey, U.K. Benzyl Viologen, Methyl Viologen, 2,6-dichlorophenol-indophenol were from BDH. Diquat bromide and Morfamquat dichloride were generous gifts from the Plant Protection Division, Imperial Chemical Industries Ltd., Bracknell, Berks. RG12 6EY, U.K. *N*-Methylphenazonium-3-sulphonate and 2,6-dichlorophenol-indophenol-3-sulphonate were kindly given by Dr. W. Draber, Bayer A.G., Wuppertal, West Germany, and also by Dr. G. Hauska, Lehrstuhl für Biochemie der Pflanzen, Rühr-Universität Bochum, West Germany. Dr. W. Draber also provided dibromothymoquinone. Vitamin-free casamino acids were purchased from Difco Laboratories, Detroit, MI, U.S.A. Tryptone and yeast extract were purchased from Oxoid Ltd., London S.E.1, U.K. Carbonyl cyanide *m*-chlorophenylhydrazone was from Calbiochem Ltd., Hereford HR4 9BQ, U.K., nigericin from Eli Lilly and Co., Indianapolis, IN 46206, U.S.A., and sodium tetraphenylboron from Koch–Light Laboratories Ltd., Colnbrook SL3 0BZ, Bucks., U.K.

Results

Uptake of oxidized bipyridylium and bipyridylium radicals by cells of E. coli

When cells of strain A1004a (haem-deficient), derepressed for nitrate reductase, were incubated with oxidized Methyl Viologen, less than 15% of the Methyl Viologen was recovered in the cell pellet after centrifugation. Oxidized Benzyl Viologen (16%) and Diquat (5%) behaved similarly. Morfamquat was not tested. These values were uninfluenced by the presence of 1mM-KNO_3 in the cell suspension. If the bipyridylium in the cell suspension was first reduced to the radical and then reoxidized with NO_3^-, H_2O_2 or air, the recovery of Diquat (5%) or Methyl Viologen (14%) in the cell pellet was unchanged, whereas that of Benzyl Viologen was much increased (90%). The radical of Benzyl Viologen behaves as if it enters the cell, but its oxidized species does not. According to this interpretation the cells became loaded with Benzyl Viologen by exposure to the radical, which diffuses across the membrane, followed by oxidation of the radical to the oxidized species, which cannot diffuse out of the cell and is therefore trapped. It was noted that dithionite was not an effective reductant of oxidized Benzyl Viologen trapped in cells unless the cells were first disrupted by sonication. This suggests that dithionite does not cross the membrane of *E. coli*, which is consistent with reports that dithionite does not cross two other types of membrane, that of erythrocytes (Keilin & Hartree, 1946) and that formed by phospholipid vesicles (Kimmelberg & Lee, 1970).

Nitrate reductase activity with bipyridylium radicals as electron donors

Cells that had been de-repressed for nitrate reductase synthesis by anaerobic growth in a medium containing glycerol and nitrate exhibited a fast nitrate-dependent oxidation of Benzyl Viologen radical, and we ascribe this activity to nitrate reductase. In this assay with whole cells, Diquat radical was also an effective electron donor to nitrate, but the radical of Methyl Viologen was only slowly oxidized and that of Morfamquat scarcely at all (Table 2). Essentially similar results were obtained with spheroplasts in place of cells, provided that the assay medium also contained 0.5M-sucrose as osmotic support. If the cells were broken by sonication the nitrate reductase activity measured with Benzyl Viologen radical was increased by about 50% or less. The activity with Diquat radical was increased approx. twofold, with Methyl Viologen radical eightfold, and with Morfamquat radical 20-fold (Table 2). Essentially similar effects were observed when spheroplasts were assayed in hypo-osmotic medium lacking adequate osmotic support. The inclusion in the assay medium of the sodium salt (0.01mM) of the membrane-penetrating anion tetraphenylboron (Skulachev, 1971) did not alter the rates of bipyridylium-radical oxidation by nitrate in these experiments. Suitable control experiments showed that the differing behaviours of the bipyridylium radicals (Table 2) were not due to the differences in associated anion (chloride or bromide,

Table 2. *Rates of oxidation of bipyridylium radicals by nitrate in intact and broken cells*

The cells used for nitrate-reduction studies were from either strain A1004a grown without 5-aminolaevulinate, giving haem-deficient cells, or the parent strain A1002, which is not haem-deficient. Growth conditions and assay procedures are described in the Materials and Methods section. Rates are expressed as the mean ± S.E.M., with the number of assays in parentheses. Each mean is that for the intact or broken cells of a single culture. The absolute values of the measured activities varied by about twofold over studies involving several cultures, although the relative effects of cell breakage, haem deficiency and variation of reductant did not alter significantly. Nitrate reductase activities are expressed as μmol of bipyridylium radical oxidized/min per mg of cell protein. Cells were broken by sonication.

	Nitrate reductase activity			
	Whole cells		Broken cells	
Radical	A1002	A1004a	A1002	A1004a
Benzyl Viologen	1.88 ± 0.24 (4)	4.46 ± 0.2 (4)	2.68 ± 0.18 (4)	5.26 ± 0.32 (4)
Diquat	1.22 ± 0.16 (4)	<0.1	1.90 ± 0.18 (4)	3.36 ± 0.40 (5)
Methyl Viologen	0.40 ± 0.16 (8)	0.24 ± 0.02 (4)	3.42 ± 0.34 (8)	3.28 ± 0.50 (5)
Morfamquat	<0.1	<0.1	1.5 ± 0.34 (4)	2.12 ± 0.38 (5)

see Table 1 above). The most straightforward interpretation of these data is that the radicals of Methyl Viologen and Morfamquat are prevented by the cytoplasmic membrane from donating electrons to nitrate reductase, whereas the radicals of Diquat and Benzyl Viologen are not. Although it can be concluded that the radicals of Methyl Viologen and Morfamquat must cross the cytoplasmic membrane to react directly or indirectly with nitrate reductase, this cannot be concluded for the radicals of Diquat and Benzyl Viologen, at least on the evidence available from Table 2.

When the haem-less strain A1004a was used after growth in the absence of 5-aminolaevulinate, nitrate reductase was unable to accept electrons from the dehydrogenases or the cytochrome-less residue of the respiratory chain (Kemp *et al.*, 1975). Nitrate reductase is to a variable extent present in the cytoplasm rather than in the cytoplasmic membrane when cytochrome synthesis is absent (MacGregor, 1975; R. W. Jones, unpublished work). Haem deficiency altered the reactivity of bipyridylium radicals with nitrate reductase in one important respect; Diquat radical was no longer an effective electron donor in cells or spheroplasts (Table 2). We conclude that Diquat radical is oxidized by nitrate reductase by two pathways, one direct and the other indirect. The direct pathway involves only nitrate reductase, is independent of cytochrome *b*, and is seen only if the permeability barrier imposed by the cytoplasmic membrane is broken. The indirect pathway involves cytochrome *b* and receives its electrons from Diquat radical at the outer aspect of the cytoplasmic membrane. The direct pathway is reactive with all four bipyridylium radicals tested. Only Diquat reacts with the indirect pathway.

Spectrophotometric determination of the sites at which bipyridylium radicals donate electrons to the nitrate-reduction pathway

An alternative to the uptake method (see above) for determining the site at which bipyridylium radicals were oxidized was developed on the assumption that dithionite is an impermeant anion for *E. coli*, as it is for erythrocytes (Keilin & Hartree, 1946). The experimental design is illustrated by the spectrophotometric recording of Fig. 1(*a*). Initially a cuvette was set up containing oxidized Benzyl Viologen and cells in buffer. The oxidized Benzyl Viologen was then reduced, with just sufficient dithionite, to the coloured radical, which could diffuse across the cytoplasmic membrane and presumably distribute itself according to the membrane potential. Aeration of the cuvette caused rapid oxidation of the radical to its non-permeant oxidized form. Subsequently the addition of excess of dithionite reduced the Benzyl Viologen to its radical in two distinct phases: a fast phase complete with the 2–3 s magnetic-mixing time, and a slow phase proceeding with a half-time of 2–3 min. If further oxidized Benzyl Viologen was added during the slow phase, it was reduced completely to its radical in 2–3 s, showing the effectiveness of the dithionite in the bulk phase. If in this experiment sonicated cells replaced intact cells, only a fast and complete phase of reduction of Benzyl Viologen to its radical was observed (Fig. 1*b*). When Diquat replaced Benzyl Viologen, only the fast phase was apparent (Fig. 1*c*). The different behaviour of Benzyl Viologen and Diquat was unaltered when spheroplasts with osmotic support replaced intact cells. These observations show that dithionite, oxidized Diquat, oxidized

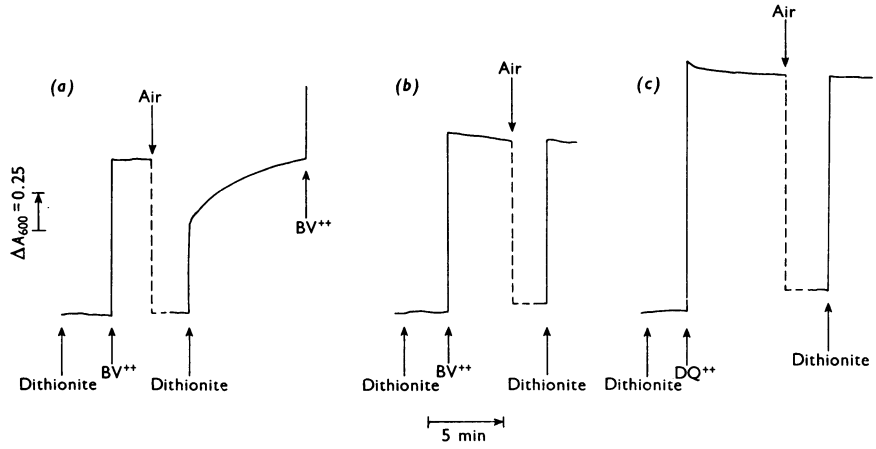


Fig. 1. *Spectrophotometric measurements of the reduction by dithionite of intra- and extra-cellular oxidized Benzyl Viologen* The reduction of oxidized Benzyl Viologen to its coloured radical was measured spectrophotometrically at 600nm in an anoxic cuvette, as described in the Materials and Methods section. Whole cells of parental strain A1002 grown anaerobically in a glycerol medium with nitrate were used for trace (*a*) at a cell protein concn. of 0.5mg/ml. In trace (*b*) the cells had been broken by sonication. In trace (*a*) the following further additions (with abbreviation and final concn. in parentheses) were made: $Na_2S_2O_4$ (dithionite, 2.0mM), oxidized Benzyl Viologen (BV^{++}, 0.16mM), oxygen (air, introduced by removing the stopper of the cuvette and taking the contents repeatedly into a Pasteur pipette until the colour of Benzyl Viologen radical had disappeared), $Na_2S_2O_4$ (dithionite, 2.0mM), oxidized Benzyl Viologen (BV^{++}, 0.05mM). In trace (*b*) the same abbreviations and concentrations apply. When air was being admitted, the spectrophotometric output was not recorded. Trace (*c*) shows an experiment using whole cells and additions of $Na_2S_2O_4$ (2mM), oxidized Diquat (DQ^{++}, 0.35mM), air and $Na_2S_2O_4$ (2mM) as shown at the arrows. The appearance of Diquat radical was measured at 460nm.

Benzyl Viologen and Diquat radical do not cross the cytoplasmic membrane of *E. coli*, whereas Benzyl Viologen radical does.

Fig. 2 shows experiments similar in principle to those of Fig. 1, except that small amounts of KNO_3 were used to oxidize the bipyridylium radicals by nitrate reductase activity. The subsequent response of the oxidized bipyridylium to dithionite was used to determine whether the nitrate-oxidized bipyridylium was intra- or extra-cellular. The results were very clear and show that Benzyl Viologen radical was oxidized by nitrate reductase intracellularly (Figs. 2*a* and 2*b*), whereas Diquat radical was oxidized extracellularly (Fig. 2*c*). Morfamquat radical was oxidized too slowly by nitrate and whole cells for study by this approach, but the slow rate of oxidation of Methyl Viologen radical was shown to be intracellular (Fig. 2*d*).

Impermeability of spheroplasts to dithionite shown by osmotic-swelling experiments

Fig. 3 shows the result of osmotic-swelling experiments with spheroplasts suspended in iso-osmotic solutions of the solutes under study (Sistrom, 1958). Fig. 3(*a*) shows a control experiment in which spheroplasts were suspended in 85mM-K_2SO_4/125mM-sodium acetate. The spontaneous rate of swelling is probably due to entry of acetic acid followed by exchange of H^+ for Na^+ by the Na^+/H^+ antiporter (West & Mitchell, 1974). The further addition of nigericin accelerates the rate of swelling, presumably by exchange of H^+ (from acetic acid within the spheroplasts) for K^+. When 85mM-$Na_2S_2O_4$ replaced sodium acetate, there was no spontaneous swelling, nor could swelling be induced by the addition of nigericin, which would permit swelling if $H_2S_2O_4$ (if it exists) entered (Fig. 3*b*). Nor did the further addition of the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone cause swelling, even though this ionophore in the presence of nigericin would permit movement of K^+ to follow any movement of $HS_2O_4^-$ or $S_2O_4^{2-}$ into the spheroplasts. It is concluded that dithionite does not diffuse readily across the cytoplasmic membrane of *E. coli*.

Effects of respiratory inhibitors on the oxidation of bipyridylium compounds by nitrate

The respiratory inhibitors 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (Cox *et al.*, 1970) and dibromothymoquinone (Poole & Haddock, 1975) inhibited the nitrate-dependent oxidation of Diquat by intact spheroplasts of the parent strain A1002 (Fig. 4). The degree of inhibition was less if the spheroplasts were osmotically disrupted, and disappeared altogether if

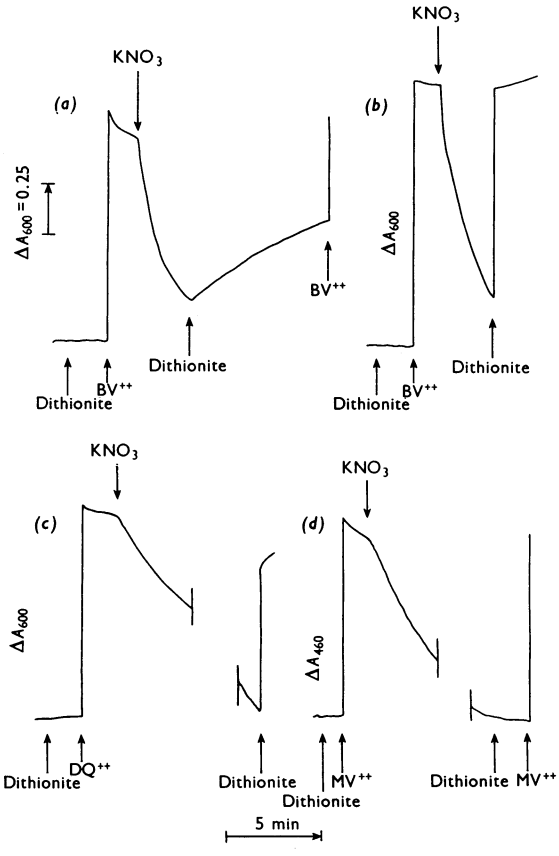


Fig. 2. *Spectrophotometric determination of the sites of nitrate-dependent oxidation of bipyridylium radicals by nitrate reductase activity of cells*

Cells of parental strain A1002 were grown anaerobically in a glycerol medium with nitrate. Spectrophotometric measurements of bipyridylium radical were made as in Fig. 1. The measuring wavelength was 600nm for traces (*a*), (*b*) and (*d*), and 460nm for trace (*c*). Additions were made as shown in the Figure, and were $Na_2S_2O_4$ (dithionite, 2.0mM), oxidized bipyridylium [traces (*a*) and (*b*), Benzyl Viologen (BV^{++}, 0.17mM); trace (*c*), Diquat (DQ^{++}, 0.37mM); trace (*d*), Methyl Viologen (MV^{++}, 0.1mM)]. In trace (*b*) the cells had been disrupted by sonication; otherwise intact cells were used.

disrupted spheroplasts of haem-deficient strain A1004a were used. These inhibitors at the concentrations used for the experiments of Fig. 4 did not inhibit the nitrate-dependent oxidation of Benzyl Viologen radical by either strain. These observations indicate that the direct, cytochrome-independent pathway of nitrate reduction by bipyridylium radicals (see above) is insensitive to the respiratory inhibitors, whereas the indirect, cytochrome *b*-dependent Diquat-specific pathway is.

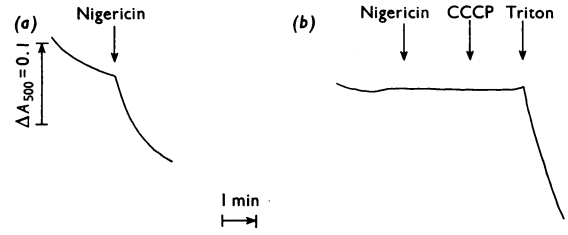


Fig. 3. *Osmotic swelling of spheroplasts*

Spheroplasts were prepared from cells of strain A1002 grown aerobically on a glycerol medium, and were suspended (3.6mg of protein) in 2.0ml of either (trace *a*) 85mM-K_2SO_4/125mM-sodium formate or (trace *b*) 85mM-K_2SO_4,/85mM-$Na_2S_2O_4$ at 22°C, in a spectrophotometer cuvette of 1.0cm light-path. Swelling was measured as a decrease in A_{500}. Further additions added as shown in the Figure were nigericin (5μg), CCCP (carbonyl cyanide *m*-chlorophenylhydrazone, 9nmol) and Triton (Triton X-100, 100μg).

Oxidation of bipyridylium radicals by nitrite

Since the product of nitrate reductase is nitrite, it was appropriate to inquire whether bipyridylium radicals could donate electrons to a nitrite reductase activity. Table 3 shows that they can, although only with Morfamquat radical did cell breakage significantly increase the nitrite reductase activity. It can be concluded from Table 3 that strain A1002 possesses at least one nitrite reductase activity capable of accepting electrons from bipyridylium radicals. The high reactivity with Diquat and Methyl Viologen radicals indicates that this nitrite reductase activity is located at the periplasmic side of the cytoplasmic membrane. Benzyl Viologen radical also donates electrons to this periplasmic side, because the oxidized Benzyl Viologen so produced is not trapped within the cell (Fig. 5).

Reactivity of some other non-permeant reductants with nitrate reductase

The non-permeant dye *N*-methylphenazonium 3-sulphonate has been successfully used to locate the sidedness of reactions in chloroplasts (Hauska, 1972). Unfortunately it was not very useful with nitrate reductase of *E. coli*, nor was dithionite. Table 4 shows that the lipid-soluble *N*-methylphenazonium ethosulphate was an effective donor to nitrate reductase, mediating electron transfer from ascorbate. Use of the haem-deficient strain A1004a showed that electron flow from *N*-methylphenazonium ethosulphate was cytochrome-dependent. *N*-Methylphenazonium 3-sulphonate was not an effective mediator between ascorbate and nitrate reductase in either whole or broken cells of strain A1002, and separate experiments (results not shown) showed that

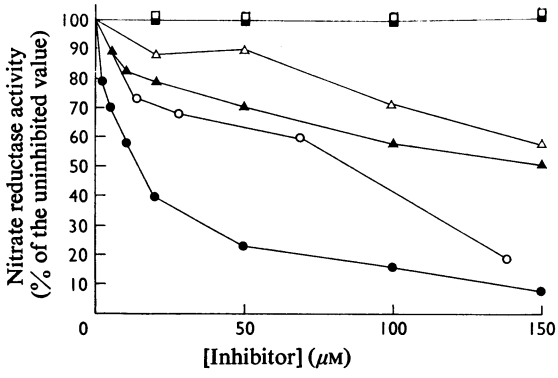


Fig. 4. *Effects of respiratory inhibitors on the oxidation of Diquat radical by nitrate*

Strain A1002 and haem-less strain A1004a were grown anaerobically in a glycerol+nitrate medium as described in the Materials and Methods section, and converted into spheroplasts as described by Garland *et al.* (1975) to remove the permeability barrier to hydrophobic molecules set by the outer layers of the cell envelope. Nitrate reductase was assayed spectrophotometrically by using Diquat radical as electron donor. Inhibitors were added with micro-syringes from an ethanolic stock solution (1 mg/ml) of 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (●, ▲, ■) and a methanolic solution (10 mM) of dibromothymoquinone (○, △, □). The latter inhibitor was in its reduced form under the conditions of the assay. Osmotic support was provided by 0.5 M-sucrose. ●, ○, Intact spheroplasts of parental strain A1002. ▲, △, Spheroplasts of strain A1002 disrupted by sonication. ■, □, Spheroplasts of haem-deficient strain A1004a disrupted by sonication. The absolute rates of nitrate reductase activity (per mg of protein) were 1.15, 2.04 and 3.11 μmol of Diquat radical oxidized/min for intact spheroplasts of strain A1002, disrupted spheroplasts of strain A1002 and disrupted spheroplasts of strain A1004a respectively. No inhibititon by 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide or dibromothymoquinone was observed when Benzyl Viologen radical was substituted for Diquat radical.

Table 3. *Rates of oxidation of bipyridylium radicals by nitrite*

Cells of strain A1002 were grown in a glycerol medium with nitrate. The means ± S.E.M. for four separate assays are given for each radical. Essentially similar results were obtained with three further cultures.

	Rate of radical oxidation (μmol/min per mg cell protein)	
Radical	Whole cells	Broken cells
Benzyl Viologen	0.29 ±0.05	0.32±0.03
Diquat	0.72 ±0.12	0.43±0.18
Methyl Viologen	0.47 ±0.04	0.34±0.03
Morfamquat	0.025±0.004	0.15±0.005

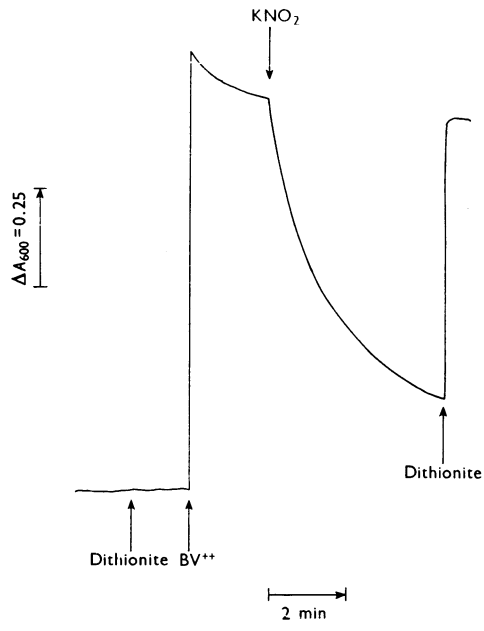


Fig. 5. *Nitrite-dependent oxidation of Benzyl Viologen radical by cells of strain A1002*

Cells were grown anaerobically in a glycerol medium with nitrate. The spectrophotometric assay conditions were exactly as for Fig. 2(*a*), except that KNO_3 was replaced by KNO_2 (15 μM).

the rate-limiting step was not the oxidation of ascorbate by the dye. Dithionite was not an effective reductant for nitrate reductase; it gave a rate of nitrate reduction that was only a few per cent of that obtained with bipyridylium radicals.

Fumarate-dependent oxidation of bipyridylium radicals

Table 5 shows that cells of strain A1002 grown anaerobically in a medium containing fumarate carried out a fumarate-dependent oxidation of bipyridylium radicals, but, except for Benzyl Viologen, only if the cells were broken. These observations indicate that the bipyridylium radicals must cross the cytoplasmic membrane before donating their electrons to the pathway of fumarate reduction. The threefold stimulation of the rate of Benzyl Viologen oxidation resulting from cell breakage can be attributed to removal of fumarate transport (Kay & Kornberg, 1971) as a rate-limiting step. The rate of Benzyl Viologen radical entry into *E. coli* is not rate-limiting for fumarate reduction (Table 2). The experiment of Fig. 6 shows that the oxidized Benzyl Viologen formed by fumarate-dependent oxidation of the radical in whole cells is inaccessible to added dithionite, showing that the radical was oxidized by the fumarate reductase pathway at an intracellular site.

Table 4. *Rates of nitrate reduction with some alternative donor systems*

Cells of strain A1002 were grown in a glycerol medium with nitrate. Cells of strain A1004a (haem-deficient) were grown in a glucose medium with nitrate. Rates are given as means ± S.E.M., with the number of separate assays in parentheses. n.t., Not tested. The absolute values of the measured activities varied by about twofold over three further cultures, but the relative effects of cell breakage, haem deficiency or choice of reductant did not vary significantly.

	Nitrate reductase activity (μmol/min per mg of cell protein)			
	Whole cells		Broken cells	
Reductant	A1002	A1004a	A1002	A1004a
Ascorbate + *N*-methylphenazonium ethosulphate	1.32 ± 0.27 (5)	0.17 ± 0.12 (4)	1.53 ± 0.29 (4)	0.13 ± 0.05 (4)
Ascorbate + *N*-methylphenazonium-3-sulphonate	<0.05	n.t.	<0.05	n.t.
Dithionite (5 mM)	0.031 ± 0.003 (6)	n.t.	0.072 ± 0.008 (5)	n.t.
Dithionite (5 mM) + Benzyl Viologen (0.1 mM)	0.92 ± 0.16 (6)	n.t.	1.53 ± 0.27 (6)	n.t.

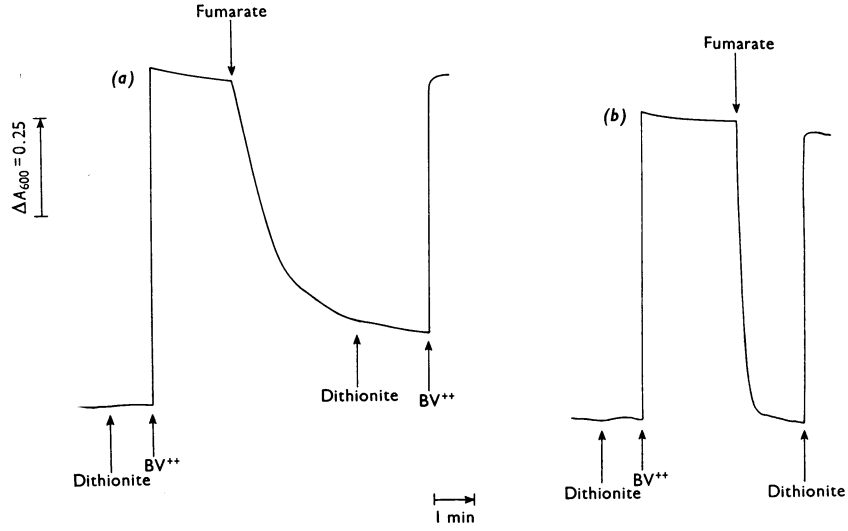


Fig. 6. *Spectrophotometric demonstration of the site at which nitrite-dependent oxidation of Benzyl Viologen radical occurs with cells of strain A1002*

Cells were grown anaerobically in a glycerol and fumarate medium. The experimental conditions for the spectrophotometric experiments were as for Fig. 2(*a*), except that 1 mM-sodium fumarate replaced KNO_3. Cells were used either intact [trace (*a*)] or ultrasonically disrupted [trace (*b*)].

Table 5. *Rates of oxidation of bipyridylium radicals by fumarate*

Cells of strain A1002 were grown in a glycerol medium with fumarate. The means ± S.E.M. for four separate assays for each radical are given for a single culture. Several other cultures gave similar results.

	Rate of radical oxidation (μmol/min per mg of cell protein)	
Radica	Whole cells	Broken cells
Benzyl Viologen	0.08 ± 0.01	0.25 ± 0.02
Diquat	<0.03	0.35 ± 0.001
Methyl Viologen	<0.03	0.56 ± 0.01
Morfamquat	<0.03	0.07 ± 0.03

Location of the sites at which formate enzymically reduces artificial acceptors

There are two distinct enzymic activities in *E. coli* that can effect the reduction of artificial acceptors by formate. One, formate dehydrogenase, preferentially reduces *N*-methylphenazonium dyes, ferricyanide and Methylene Blue (Enoch & Lester, 1975). The other, formate hydrogenlyase, preferentially reduces Benzyl Viologen. The growth conditions required for the appearance of these two activities differ in each case (Lester & De Moss, 1971; Ruiz-Herrera & Alvarez, 1972). Garland *et al.* (1975) concluded that formate reacted with formate dehydrogenase at the

Table 6. *Rates of reduction of artificial acceptors by formate*

Rates are expressed as μmol of acceptor reduced/min per mg of cell protein. Strain A1002 was used, and grown anaerobically on glycerol with nitrate medium to induce formate dehydrogenase activity '(formate dehydrogenase cells'), or on a glycerol+fumarate+formate medium to induce the synthesis of formate hydrogenlyase ('formate hydrogenlyase cells'). Abbreviations: PMS, *N*-methylphenazonium methosulphate; PMS-S, *N*-methylphenazonium-3-sulphonate (Hauska, 1972). DCPIP, 2,6-Dichlorophenol-indophenol; DCPIP-S, its 3-sulphonate. The concentrations of dyes are given in the Materials and Methods section, as are other experimental conditions. Similar results were obtained with the three further pairs of cultures.

	Formate dehydrogenase cells		Formate hydrogenlyase cells	
Acceptor	Intact cells	Broken cells	Intact cells	Broken cells
Benzyl Viologen	0.038±0.003 (6)	0.036±0.008 (6)	0.17 ±0.02 (5)	0.04 ±0.002 (4)
Diquat	0.096±0.028 (7)	0.085±0.009 (7)	0.29 ±0.01 (5)	0.042±0.006 (4)
Methyl Viologen	0.008±0.001 (4)	0.007±0.001 (4)	0.13 ±0.03 (5)	n.t.
Morfamquat	0.016±0.003 (5)	0.018±0.001 (5)	0.23 ±0.03 (5)	0.09 ±0.02 (4)
PMS+DCPIP	0.42 ±0.07 (6)	0.46 ±0.07 (7)	0.058±0.005 (5)	0.057±0.006 (5)
PMS-S+DCPIP	0.005±0.002 (5)	0.021±0.003 (6)	n.t.	n.t.
PMS+DCPIP-S	0.65 ±0.07 (5)	0.42 ±0.05 (6)	0.163±0.024 (7)	0.137±0.006 (7)
Ferricyanide	<0.002	0.021±0.008 (8)	0.016±0.002 (4)	0.035±0.005 (6)

cytoplasmic aspect of the cytoplasmic membrane, but otherwise information on the sites at which dyes are enzymically reduced by formate is lacking. Table 6 shows the results of experiments where the rate of reduction of artificial acceptors by formate was compared in intact and broken cells of strain A1002 grown anaerobically under conditions appropriate for the appearance of either formate dehydrogenase or formate hydrogenlyase. It was observed that the rate of reduction of oxidized bipyridylium compounds by formate in cells induced for formate hydrogenlyase was high. Evidence was presented above that these oxidized bipyridylium compounds do not diffuse across the cytoplasmic membrane of *E. coli*, so we conclude that they are reduced by formate hydrogenlyase on the periplasmic aspect of that membrane. The loss of formate hydrogenlyase activity after cell breakage (Table 6) is unexplained. As expected, neither intact nor broken cells induced for formate dehydrogenase by growth in a glycerol+nitrate medium exhibited significant formate-dependent rates of reduction of oxidized Benzyl Viologen, whereas *N*-methylphenazonium was rapidly reduced. The hydrophilic derivative *N*-methylphenazonium-3-sulphonate was a poor acceptor for formate dehydrogenase in both intact and broken cells, although the fourfold stimulation of activity observed on cell breakage favours the conclusion that this acceptor is reduced on the cytoplasmic aspect of the cytoplasmic membrane. Confirmation of this conclusion comes from the observation that the reduction of non-permeant ferricyanide (Futai, 1974) by formate in formate dehydrogenase was at least tenfold stimulated by cell breakage (Table 6).

Discussion

Differing reactivities of bipyridylium radicals with oxidoreduction enzymes of E. coli

Two factors other than oxidoreduction potential determine the reactivity of a given bipyridylium radical with oxidoreduction enzymes in *E. coli*. One is the ability or otherwise of the radical to cross the cell membrane, and is clearly illustrated by the results with nitrate reductase (Table 2) and fumarate reductase (Table 5) activities. All of the radicals used were water-soluble, and comparison of spheroplasts with whole cells showed that the outer layers of the cell envelope did not present a permeability barrier to the radicals. The other factor determining reactivity is chemical structure, and this was well illustrated by the ability of Diquat radical, a 2,2′-bipyridylium (Table 1), to react indirectly with nitrate reductase through an inhibitor-sensitive cytochrome-*b* dependent pathway, whereas the 4,4′-bipyridylium radicals did not (Table 2, Figs. 2 and 4). A wide range of bipyridylium compounds can be synthesized, and they may prove useful in exploring the topography of membrane-bound oxidoreduction enzymes.

Transmembrane orientation of the nitrate reductase pathway

The anaerobic respiratory nitrate reductase pathway of *E. coli* is proton-translocating, and the topography of the enzymes concerned has special significance for the mechanism of proton translocation (Mitchell, 1966). Our finding that nitrate reductase can be reduced directly from the inner aspect of the cytoplasmic membrane and also indirectly from the

outer aspect by a cytochrome *b*-dependent pathway shows that the electron-transfer pathway leading to nitrate reductase spans the membrane not only structurally (Boxer & Clegg, 1975) but also functionally.

Identity of the Diquat-specific acceptor for nitrate reduction

Positive identification of the periplasmic facing site at which Diquat radical indirectly reduces nitrate reductase by a cytochrome *b*-dependent pathway that is sensitive to respiratory inhibitors remains an important task. This Diquat-specific site does not include any of the components of the fumarate reductase pathway, because fumarate does not oxidize Diquat radical in whole cells (Table 5), whereas nitrate does (Table 2). Thus menaquinone (Newton *et al.*, 1971) is not reduced by Diquat radical, at least from the periplasmic aspect of the cytoplasmic membrane, and neither is the NADH dehydrogenase. Nor does it seem likely that the hydrophilic membrane-impermeant Diquat radical could directly reduce the ubiquinone of the membrane when it could not reduce the menaquinone. The specificity of the reactivity of Diquat radical is best explained by postulating that the electron acceptor is a protein able to discriminate between the 2,2′-bipyridylium radical, which is a stiff planar molecule, and the more flexible 4,4′-bipyridylium radicals such as Methyl Viologen or Morfamquat. An obvious candidate for the Diquat-specific site is cytochrome *b* of the nitrate reductase pathway, already known to be accessible on the periplasmic face of the cytoplasmic membrane (Boxer & Clegg, 1975).

Location of other oxidoreduction enzymes in relation to the cytoplasmic membrane

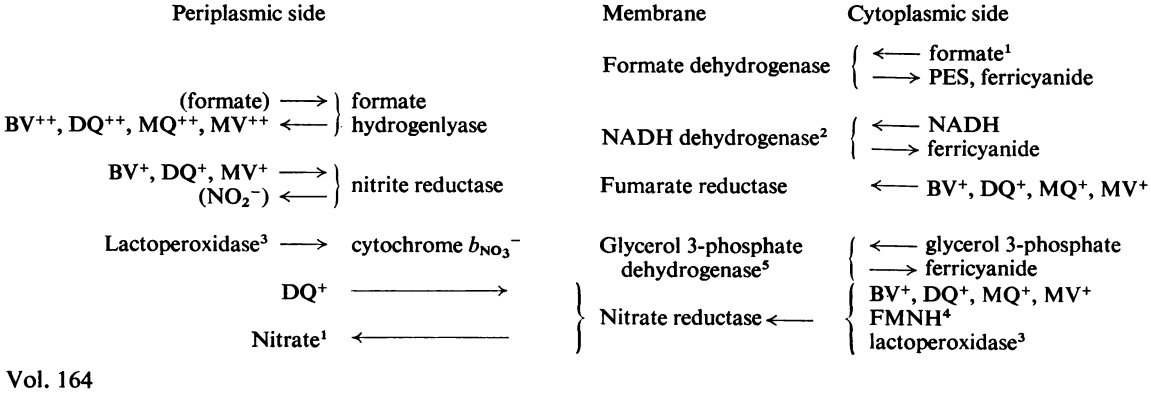
A knowledge of the relationship of oxidoreduction enzymes to the cytoplasmic membrane is relevant to the physiology and mechanism of the oxidoreductions and also to any processes that they may drive, such as proton translocation and solute transport. Table 7 summarizes the relationship of a number of these oxidoreduction enzymes to the cytoplasmic membrane of *E. coli*. Most of these enzymes can be located only on the inner cytoplasmic aspect of the membrane, and thus the identification of an externally placed cytochrome *b*, probably identical with the Diquat-specific site for nitrate reduction, is of considerable significance in support of the idea that respiratory oxidoreductions must be transmembranous to drive proton translocation (Mitchell, 1966).

Electroneutrality requirements for the diffusion of Benzyl Viologen radical across the cytoplasmic membrane

When Benzyl Viologen radical crosses the cytoplasmic membrane of *E. coli* its movement would rapidly be terminated by a diffusion potential unless

Table 7. *Relationship of some oxidoreduction enzymes to the cytoplasmic membrane of E. coli*

The Table lists enzyme activities in two columns according to whether the electron donor or acceptor given is at the periplasmic or cytoplasmic side of the cytoplasmic membrane. Where the site at which a substrate reacts with an enzyme has not been demonstrated, that substate is placed in parentheses. Accessibility to iodination by lactoperoxidase is also shown (Boxer & Clegg, 1975). Abbreviations: BV, Benzyl Viologen; DQ, Diquat; MQ, Morfamquat; MV, Methyl Viologen; with positive charges to indicate their oxidoreduction state; PES, *N*-methylphenazonium ethosulphate and its sites of reaction are assumed to be as for *N*-methylphenazonium-3-sulphonate. References: [1]Garland *et al.* (1975); [2]Futai (1974); [3]Boxer & Clegg (1975); [4]Kemp *et al.* (1975); [5]Weiner (1974). Otherwise information is from the Results section of the present paper. No distinction is made between a side of the membrane and the bulk phase adjacent to it. The arrows show the direction of electron flow, except for lactoperoxidase, where they indicate iodination.



some compensating charge movement occurred, such as extrusion of H^+ or K^+ or uptake of an anion; we have no information on this point. Conceivably Benzyl Viologen radical could be used as a permeant cation whose distribution would permit calculation of the membrane potential. It seems unlikely that Benzyl Viologen radical entered the cell indirectly as the uncharged fully reduced species, for not only would the concentration of this neutral species be very low, it would also require an intracellular oxidant to obtain the extent of intracellular accumulation of Benzyl Viologen radical observed in the experiment of Fig. 1(*a*), where neither nitrate nor fumarate was provided. Also to postulate that the electroneutral fully reduced bipyridylium is the permeant species would leave unexplained the inability of Diquat radical to cross the membrane, despite the fact that the radical must provide by reproportionation at least some concentration of fully reduced Diquat.

Inability of dithionite to cross the cytoplasmic membrane of E. coli

It is 30 years since Keilin & Hartree (1946) described the inability of dithionite to cross the membrane of erythrocytes, yet surprisingly little use has been made of this observation in exploring the topography of oxidoreduction enzymes in membrane vesicles (but see Kimmelberg & Lee, 1970). The inability of dithionite to cross the cytoplasmic membrane of *E. coli* is presumably paralleled by mitochondria, where it is already known that SO_4^{2-}, SO_3^{2-} and $S_2O_3^{2-}$ do not diffuse across the inner membrane (Crompton *et al.*, 1974*a*,*b*), although they may be accepted by a phosphate- or dicarboxylate-anion-transport system.

Wider implications of the permeability properties of the oxidized and radical species of bipyridylium radicals

Methyl and Benzyl Viologen are commonly used as mediators for potentiometric oxidoreductions of membrane-bound respiratory or photosynthetic enzymes (Dutton & Wilson, 1974). In this technique the mediating dye is assumed to effect equilibration of the oxidoreduction potential sensed by a platinum electrode in the bulk phase with the oxidoreduction potential of a spectroscopically observed component in the membrane phase. It is clear that the differing charges and lipophilicity of the oxidized and radical species of any bipyridylium mediator will ensure that their electrochemical activities at a membrane site will respond in different ways or extents to local or microscopic effects such as surface charge and hydrophobicity. A further complication arises when the radical but not the oxidized bipyridylium species can diffuse across the membrane, as is the case with Benzyl Viologen, and perhaps also Methyl Viologen in the long time-scale of potentiometric titrations (see Fig. 2*d* for evidence that Methyl Viologen radical can slowly cross the membrane of *E. coli*). Under these conditions the oxidoreduction potential within a membrane vesicle would bear little relation to that in the bulk phase, and would be altered by any factors that change the membrane potential (Jones *et al.*, 1976).

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