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ROLE OF QUINONES IN ELECTRON TRANSPORT TO OXYGEN AND NITRATE IN *ESCHERICHIA COLI*

STUDIES WITH A *ubiA*$^-$ *menA*$^-$ DOUBLE QUINONE MUTANT

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SUMMARY

A *ubiA*$^-$ *menA*$^-$ double quinone mutant of *Escherichia coli* K12 was constructed together with other isogenic strains lacking either ubiquinone or menaquinone. These strains were used to study the role of quinones in electron transport to oxygen and nitrate. Each of the four oxidases examined (NADH, D-lactate, α-glycerophosphate and succinate) required a quinone for activity. Ubiquinone was active in each oxidase system while menaquinone gave full activity in α-glycerophosphate oxidase, partial activity in D-lactate oxidase but was inactive in NADH and succinate oxidation. The aerobic growth rates, growth yields and products of glucose metabolism of the quinone-deficient strains were also examined. The growth rate and growth yield of the *ubi*$^+$*menA*$^-$ strain was the same as the wild-type strain, whereas the *ubiA*$^-$*men*$^+$ strain grew more slowly on glucose, had a lower growth yield (30 % of wild type) and accumulated relatively large quantities of acetate and lactate. The growth of the *ubiA*$^-$*menA*$^-$ strain was even more severely affected than that of the *ubiA*$^-$*men*$^+$ strain.

Electron transport from formate, D-lactate, α-glycerophosphate and NADH to nitrate was also highly dependent on the presence of a quinone. Either ubiquinone or menaquinone was active in electron transport from formate and the activity of the quinones in electron transport from the other substrates was the same as for the oxidase systems. In contrast, quinones were not obligatory carriers in the anaerobic formate hydrogenlyase system. It is concluded that the quinones serve to link the various dehydrogenases with the terminal electron transport systems to oxygen and nitrate and that the dehydrogenases possess a degree of selectivity with respect to the quinone acceptors.

Abbreviations: Q, ubiquinone; MK, menaquinone; DMK, demethylmenaquinone; DMK/MK, the naturally occurring mixture of DMK and MK formed by *Escherichia coli*; the number of isoprene units in the side-chains of the different quinone homologues is indicated by the number after the abbreviation (e.g. Q-8); CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

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INTRODUCTION

It is now generally accepted that in mitochondria ubiquinone is an essential component of the respiratory chain [1]. This conclusion is supported by extraction-reactivation experiments [2, 3] and by recent work with a ubiquinone-deficient mutant of *Saccharomyces cerevisiae* [4]. Studies of quinone function in bacteria have been less extensive [1, 5, 6]. The function of quinones in organisms like *Escherichia coli* is potentially more complex than in mitochondria since both ubiquinone and menaquinone are present, a wider range of reduced substrates is oxidized and three alternative electron transport pathways to oxygen, fumarate or nitrate may be used. In the case of aerobic electron transport in *E. coli*, previous irradiation-reactivation studies by Kashket and Brodie [7] led to the conclusion that menaquinone is involved in NADH oxidase and ubiquinone in succinate oxidase. However, Cox et al. [8], as a result of studies using a *ubiB*$^-$ mutant, have suggested that ubiquinone is required for the oxidation of NADH and also for D-lactate in *E. coli*. Cox et al. [8] proposed that ubiquinone functions together with non-haem iron at two sites, before and after cytochrome *b* in NADH oxidation and only after cytochrome *b* in D-lactate oxidation. The ubiquinone-non-haem iron sites were postulated to be directly involved in oxidative phosphorylation. Evidence in support of this proposal has been provided recently by Crane and colleagues [9, 10] who have reported that both electron transport and ATPase are inhibited by iron chelators and quinone analogues.

The role of quinones in the anaerobic nitrate reductase system of *E. coli* has not previously been studied using quinone-deficient mutants. Itagaki [11] reported that quinone-depleted membrane particles lost formate-nitrate reductase activity and that partial restoration of activity could be achieved by the addition of ubiquinone or menaquinone. More recently, it was shown that ubiquinone or menaquinone strongly stimulated formate-nitrate reductase activity in a purified system containing formate dehydrogenase and nitrate reductase [12].

In the present paper, we describe the derivation and properties of a double quinone mutant (*ubiA*$^-$*menA*$^-$) of *E. coli* K12 and related strains lacking either quinone. Using these strains we have examined the role of both ubiquinone and menaquinone in aerobic respiration and in the nitrate reductase system.

MATERIALS AND METHODS

Bacterial strains. All strains used were derivatives of *E. coli* K12. The strains carrying the *ubiA*$^-$ and *menA*$^-$ alleles were constructed as follows. The *menA401* allele was transferred into strain AN383 (*ubiA420men*$^+$) by cotransduction with the *metB* gene as described previously [13] giving strain AN384 (*ubiA420menA401*). Strain AN385 (*ubiA420men*$^+$) was a *metB*$^+$ transductant from the same transduction cross, which had not received the *menA*$^-$ allele. Strains AN386 (*ubi*$^+$*menA401*) and AN387 (*ubi*$^+$*men*$^+$) were derived from strains AN384 and AN385, respectively, by transduction to *ubi*$^+$ using PIKC grown on a wild-type strain by selecting for the ability to grow on succinate as sole carbon source.

Chemicals. Q-3 and MK-1 were kindly donated by Dr. O. Isler, F. Hoffman, La Roche and Co., Basle, Switzerland.

Media. Minimal medium has been described previously [14] and was used at

double strength. Glucose and other supplements were sterilized separately and, unless otherwise stated, were added at the following concentrations: D-glucose, 30 mM; casamino acids, 0.1 %; succinate, 30 mM; and thiamine—HCl, 1 μM. When growing cells aerobically for membrane preparations, glucose-minimal medium supplemented with casamino acids was used.

For optimal formate-nitrate reductase activity in anaerobic cells the medium contained in addition, Na_2MoO_4, 1 μM; Na_2SeO_3, 1 μM; $KHCO_3$, 20 mM; and KNO_3, 100 mM [15]. For optimal formate hydrogenlyase activity, KNO_3 was omitted [15]. The complete medium used was brain-heart infusion (Oxoid).

Growth of cells. For aerobic cultures, cells were grown at 37 °C in 10-l quantities in glass fermentors with aeration (12 l/min) and stirring (500 rev./min), and were harvested in the late exponential phase of growth. For anaerobic cultures, cells were grown at 37 °C in 10-l glass bottles fitted with inlet and outlet tubes. The bottles containing the medium were autoclaved, filled to the neck with additional medium, allowed to cool under oxygen-free nitrogen prior to inoculation and maintained under nitrogen during growth.

Strains were purified before use by streaking for single colonies on brain-heart infusion plates and the purified strains restreaked to give nearly confluent growth. The growth was harvested, and used to inoculate the 10-l quantities of minimal medium used for aerobic or anaerobic growth. In order to assist growth and minimize the chance of reversion, the inocula for strains carrying the *ubiA*$^-$ allele were prepared on brain-heart infusion plates supplemented with 4-hydroxybenzoate (1 mM) which allowed a low level of Q-8 to be formed [16].

During the subsequent growth of these strains for five generations in minimal medium, in the absence of 4-hydroxybenzoate, the ubiquinone present was reduced to an insignificant level. The proportion of *ubi*$^+$ revertants was determined by streaking samples of cultures to brain-heart infusion plates or succinate-minimal plates. Unlike the *ubi*$^-$ strains, the *ubi*$^+$ revertants grow on succinate as sole source of carbon and form large colonies on brain-heart infusion plates. Only those cultures which contained less than 2 % *ubi*$^+$ revertants were used for subsequent experiments.

Preparation of membranes. Membranes were prepared at 4 °C. Cells were harvested by centrifugation and washed once with sucrose/TES/Mg^{2+} (0.25 M sucrose, 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid, 0.02 M magnesium acetate, pH 7.5). The cells were resuspended in buffer at 1 g wet weight/3 ml buffer and disrupted using a Ribi cell fractionater at 20 000 lb/inch^2. The cell smash was centrifuged at 25 000 × *g* for 1 h to remove large wall fragments. This procedure also removes approx. 50 % of the cytoplasmic membrane as judged by the concentrations of quinones, oxidases and formate-nitrate reductase in the pellet and supernatant. The supernatant was then centrifuged at 150 000 × *g* for 3 h and the resulting pellet resuspended in a volume of buffer equivalent to one-fourth of the original volume of cell extract. This suspension (approx. 60 mg protein/ml) was used as the membrane preparation without further purification.

Estimation of oxidases in membranes. The oxidases present in respiratory membranes were estimated by measuring the rate of oxygen uptake using a Clark type oxygen electrode. The reaction mixture contained 50 μl of membranes (approx. 3 mg protein) and substrates (0.6 mM NADH, 20 mM succinate, 4 mM D-lactate, or 20 mM α-glycerophosphate) in sucrose/TES/Mg^{2+} buffer to a total volume of 2.5 ml.

Oxygen uptake due to the presence of endogenous substrates was allowed to reach a steady level before substrates were added except in the estimations for succinate oxidase in which case the substrate (succinate) was added to the reaction mixture before the membranes since dilution in the absence of succinate caused rapid inactivation of the succinate oxidase. The concentration of dissolved oxygen in sucrose/TES/Mg^{2+} buffer at 30 °C was taken to be 450 ng atoms per ml [17].

Assay for nitrate reductase. Assays were carried out at 30 °C in screw-capped tubes. The basal reaction mixture contained sucrose/TES/Mg^{2+} buffer (2.5 ml) sodium formate (20 mM), KNO_3 (20 mM) and Q-3 or MK-1 (48 μM, added in 3 μl of ethanol) when indicated. The tubes containing the reaction mixtures were kept in ice and flushed with nitrogen. Membranes (about 1 mg protein) were then added, the tube flushed again with nitrogen and then incubated at 30 °C. After incubation, for 10 and 20 min (in separate tubes), samples were withdrawn and the concentration of nitrite present was determined [18]. The activities obtained were approximately linear over 20 min and were dependent on the concentration of membranes used. No loss of nitrite was detected when membranes were incubated with nitrite in the presence of formate.

Assay for formate-hydrogenlyase. Cells were harvested, washed once with sodium phosphate buffer (0.05 M, pH 7.0), and resuspended in phosphate buffer (0.1 M, pH 7.0) at a concentration of 1 g wet weight of cells per 4 ml of buffer. The rate of hydrogen evolution with formate as substrate was measured manometrically using a Warburg apparatus. Flasks containing 0.1 ml of cell suspension and 2.8 ml of phosphate buffer (0.1 M, pH 7.0) with 0.1 ml M formate in the sidearm were flushed with nitrogen and after equilibration the reaction was started by tipping in the formate. The CO_2 produced was absorbed using 0.2 ml of 10 % KOH in the centre well of each flask. The rates of hydrogen evolution were approximately linear over a 30 min period and were completely dependent on the presence of formate.

Estimation of protein. Protein concentrations were measured by the method of Lowry et al. [19] using bovine serum albumin fraction V as standard.

Estimation of flavins. The method used has been described previously [20].

Estimation of cytochromes. Dithionite-reduced minus oxidized difference spectra were recorded at room temperature using an Aminco Chance DW2 dual wavelength spectrophotometer with a full scale deflection of 0.2 *A* and a spectral bandpass of 1 nm. The concentrations of cytochromes b_1 and *d* were measured using the following millimolar absorption coefficients and wavelength pairs: cytochrome b_1, 17.5 $mM^{-1} \cdot cm^{-1}$ at 560–575 nm and cytochrome *d* 8.5 $mM^{-1} \cdot cm^{-1}$ at 615–630 nm [21]. For the determination of cytochrome *o* the membranes were reduced with dithionite and a portion bubbled with CO for 2 min. A reduced CO minus reduced difference spectrum was recorded and concentrations calculated using a millimolar absorption coefficient of 80 $mM^{-1} \cdot cm^{-1}$ at the wavelength pair 415–430 nm [22]. In principle the cytochrome *o* values obtained by this method could be slightly low due to interference by cytochrome *d*. However, this interference was minimal as although the CO spectra showed the presence of cytochrome *d* (minor trough at 443 nm) the spectrum attributable to cytochrome *o* (peak 415 nm, trough 430 nm) predominated.

Estimation of quinones. The levels of Q-8, DMK-8 and MK-8 in membranes were estimated after extraction by a method based on the solvent extraction technique of Kröger and Klingenberg [23]. To allow the detection of low levels of quinones 9 ml

of membrane preparation was used. The membrane preparation (9 ml) was extracted with 27 ml of methanol/light petroleum (60 : 40, v/v) and the phases separated by centrifugation. The upper phase was retained and the lower phase extracted a further three times with light petroleum [14 ml]. The extracts were combined, 18 ml of an ethanolic solution of ferric chloride (5 mg $FeCl_3 \cdot 6H_2O$/ml) was added and the mixture allowed to stand 10 min at room temperature in the dark. The addition of $FeCl_3$ oxidizes any reduced quinones present. The mixture was then extracted with water (12 ml) to remove the $FeCl_3$, and the upper light petroleum phase was concentrated on a steam bath and chromatographed on silica gel plates using chloroform/light petroleum (70 : 30, v/v) as solvent. The orange-coloured Q-8 band (R_F 0.5) and the lemon-coloured DMK/MK-8 band (R_F 0.7) were eluted with ethanol and the concentrations of the respective quinones estimated spectrophotometrically. In the case of Q-8 the reduction in absorbance at 275 nm after the addition of solid $NaBH_4$ was measured [24] ($\Delta\Sigma$, 12 700). The DMK-8 was estimated by measuring the increase in absorbance at 560 nm after the addition of KOH [25] to 0.2 M final concentration ($\Delta\Sigma$, 9100). To measure MK-8, the increase in absorbance at 245 nm was determined after the addition of solid $NaBH_4$ to a sample containing 10 mM ammonium acetate buffer, pH 5 [25], the increase in absorbance attributable to DMK ($\Delta\Sigma$, 19 800) was subtracted, and the MK-8 concentration calculated ($\Delta\Sigma$, 25 800).

The levels of Q-8, DMK-8 and MK-8 in whole cells were determined by extraction of the cells with acetone in a Soxhlet apparatus as described previously [26]. The quinones were then chromatographed and estimated as described above.

Estimation of percentage of oxidized and reduced ubiquinone in membrane preparations. The oxidation of substrates was followed using an oxygen electrode to determine when the membranes were in the aerobic steady state or the anaerobic state. The electrode vessel contained 2.5 ml sucrose/TES/Mg^{2+} buffer; NADH (1.0 mM), α-glycerophosphate (4 mM) or succinate (20 mM), and membranes (5 mg protein). Samples (2 ml) from the aerobic steady state were mixed immediately with 6 ml of methanol/light petroleum (60 : 40, v/v). Samples from the anaerobic state were carefully withdrawn to minimize the introduction of air, held for 1 min in the syringe and then mixed with the methanol/light petroleum. The method of extraction of Q was similar to that described above. The mixtures were centrifuged to separate the phases and the upper phase retained. The lower phase was then extracted twice with 4 ml of light petroleum and the extracts combined. The combined extracts were evaporated to dryness under reduced pressure and the flasks flushed with oxygen-free nitrogen and stoppered. The amount of oxidized Q-8 in each sample was determined by dissolving the residues in 0.7 ml of ethanol and measuring the change in absorbance at 275 nm following the addition of $NaBH_4$ [24]. To determine the total Q-8 present, 3.6 ml of ethanolic ferric chloride ($FeCl_3 \cdot 6H_2O$, 5 mg/ml) was added to the combined light petroleum extracts and the mixtures allowed to stand at room temperature for 20 min in the dark. This procedure oxidized any reduced Q-8 which was present. The $FeCl_3$ was then removed by shaking with 2.4 ml of water, the upper phase re-extracted with 4 ml of 60 % ethanol and then evaporated to dryness and the amount of Q-8 present determined as described above. When membranes in which the Q-8 had been reduced with dithionite were tested by the above procedures all the Q-8 was recovered and was fully reduced.

Measurement of P/O ratios. Reaction mixtures contained the following: ADP,

0.16 mM; AMP, 1.6 mM; Na_2HPO_4, 3 mM; glucose, 3 mM; hexokinase (Sigma type F-300), 50 units of activity; NADH, 0.4 mM, or D-lactate, 4 mM, or α-glycerophosphate, 20 mM, or sodium succinate, 20 mM; ^{32}P (as PO_4^{3-}), $5 \cdot 10^5$–$1 \cdot 10^6$ dpm; membranes, 50 µl; and sucrose/TES/Mg^{2+} buffer to a total volume of 2.5 ml. The rate of O_2 uptake was measured at 30 °C using a Clark type oxygen electrode. A 2 ml aliquot of the reaction mixture was then added to 1 ml of cold 20 % trichloroacetic acid and the precipitated protein removed by centrifugation. Excess PO_4^{3-} was then removed by conversion to a phosphomolybdate complex (using 0.8 ml of $(NH_4)_2MoO_4$ solution, 5 % (w/v) in 2 M H_2SO_4) and extraction with 7 ml of isobutanol/benzene (1 : 1, v/v, saturated with water). The organic phase was then removed; 0.02 ml of 0.02 M KH_2PO_4 solution was added and the extraction step repeated once more. A 2.5 ml aliquot of the aqueous solution was then added to 7.5 ml of distilled water and counted in a Packard scintillation counter with settings for Cerenkov radiation.

RESULTS

Construction of a ubiA⁻menA⁻ double quinone mutant

In order to study the involvement of ubiquinone and menaquinone in the electron transport systems of *E. coli*, it was desirable to construct a strain completely lacking both quinones together with isogenic strains lacking either quinone. A double quinone mutant (*ubiD⁻menA⁻*) had previously been derived by Newton et al. [27] and used to study the role of menaquinone in the synthesis of uracil under anaerobic conditions. However, this mutant formed about 20 % of the wild-type level of ubiquinone under aerobic conditions and therefore was unsuitable for the present work.

An idea of the likely growth properties of a strain completely blocked in the specific pathways to ubiquinone and menaquinone was gained from previous work with *aroB⁻* strains (unpublished data). In these strains the common pathway of aromatic biosynthesis is blocked thus preventing the synthesis of the aromatic amino acids and vitamins including Q-8 and MK-8. It was found that such strains grew satisfactorily on glucose-minimal medium supplemented with phenylalanine, tyrosine, tryptophan and 4-aminobenzoate. Under these conditions no MK-8 was present but a low concentration of Q-8 was formed from 4-hydroxybenzoate which often contaminates samples of tyrosine. However, strains which possessed a *ubiA⁻* allele in addition to being *aroB⁻* were unable to grow unless the medium was supplemented with shikimate. With shikimate present normal levels of MK-8 were formed but no Q-8. These observations indicated that a strain which is completely blocked in the specific pathways to both ubiquinone and menaquinone would be unable to grow on glucose-minimal medium. To overcome this difficulty, use was made of the conditional nature of the *ubiA420* allele. Strains carrying this allele form no detectable ubiquinone unless a relatively high level (10^{-3} M) of the ubiquinone intermediate, 4-hydroxybenzoate, is added to the growth medium. Under these conditions up to 20 % of the wild-type level of ubiquinone is formed [16]. This level of ubiquinone is sufficient to allow the growth of a *ubiA420men⁻* strain on minimal medium. Using this property, a *ubiA⁻menA⁻* strain (AN384) was derived by transduction (see Materials and Methods), together with three other essentially isogenic strains (AN385, *ubiA⁻men⁺*; AN386, *ubi⁺menA⁻*; and AN387, *ubi⁺men⁺*). The *ubiA⁻* and *menA⁻* alleles affect the

octaprenyltransferases in ubiquinone and menaquinone biosynthesis, respectively [16, 28], so that no intermediates capable of participating in electron transfer processes are accumulated in the membranes of strains carrying these alleles.

Physiology of strains carrying the ubiA⁻ and menA⁻ alleles

In agreement with previous studies using a *ubiB*⁻ strain [8], it was found that the inability to synthesize ubiquinone (*ubiA*⁻*men*⁺) resulted in a marked decrease in the aerobic growth rate and growth yield with glucose as carbon source (Table I). In contrast, loss of menaquinone (*ubi*⁺*menA*⁻), did not affect the growth rate or yield provided that ubiquinone was present. In a strain lacking ubiquinone, however, the loss of menaquinone (*ubiA*⁻*menA*⁻) resulted in a further decrease in growth rate compared with the *ubiA*⁻*men*⁺ strain indicating that menaquinone plays a significant role in the aerobic metabolism when ubiquinone is absent. This conclusion is consistent with the finding (see below) that in *E. coli* respiratory membranes, menaquinone can function in the α-glycerophosphate and D-lactate oxidase systems.

Examination of culture supernatants by gas chromatography showed that the *ubiA*⁻*menA*⁺ strain accumulated larger amounts of lactate and acetate than the parent (*ubiA*⁺*menA*⁺) strain (Table I). In fact 65–70 % of the original glucose carbon was present as lactate and acetate in the supernatants from the *ubiA*⁻*menA*⁺ strain. Since the growth yield of the *ubiA*⁻*menA*⁺ strain is about 30 % that of the parent strain these results suggest that the slower growth rate and reduced growth yield of the *ubiA*⁻ strain are due to a decreased ability to oxidize the products of glycolysis.

TABLE I

AEROBIC GROWTH RATES, GROWTH YIELDS AND PRODUCTS OF GLUCOSE METABOLISM OF QUINONE MUTANTS

To determine generation times strains were grown on minimal medium with 30 mM glucose. To measure the products of glucose metabolism, cultures were grown on limiting glucose (6 mM) under the same conditions as for the growth yield experiments [30]. Acetate and lactate concentrations were estimated by gas chromatography [30].

Strain	Generation time (min)	Growth yield on glucose		Concentration (mM) in culture supernatant	
		2 mM	4 mM	Acetate	Lactate
AN387 (*ubi*⁺*men*⁺)	60	107	181	1.2	0.1
AN385 (*ubi*⁻*men*⁺)	180	28	54	2.9	7.1
AN386 (*ubi*⁺*men*⁻)	60	105	179	–	–
AN384 (*ubi*⁻*men*⁻)	420	–	–	–	–

Concentration of respiratory components in aerobic membranes from the quinone mutants

Respiratory membranes were prepared from the quinone mutants grown aerobically on glucose-minimal medium containing casamino acids (see Materials and Methods). Estimation of the quinone concentrations confirmed that the respiratory membranes from strains carrying *ubiA*⁻ or *menA*⁻ alleles contained no detectable Q-8 or DMK/MK-8, respectively (Table II). In the case of AN384 (*ubiA*⁻*menA*⁻) it proved impossible to grow cultures aerobically without a small amount of reversion to

TABLE II

CYTOCHROME, FLAVIN AND QUINONE CONTENTS OF AEROBIC MEMBRANES FROM THE QUINONE MUTANTS

Cytochrome a_1 was also present in these membranes but at concentrations which were too low to measure accurately.

Component	Concentration of component (nmol/mg protein)			
	AN384 (*ubi*$^-$*men*$^-$)	AN385 (*ubi*$^-$*men*$^+$)	AN386 (*ubi*$^+$*men*$^-$)	AN387 (*ubi*$^+$*men*$^+$)
FAD	0.19	0.28	0.19	0.15
FMN	0.08	0.11	0.08	0.09
Cytochrome *b*	0.18	0.19	0.27	0.27
Cytochrome *o*	0.017	0.011	0.034	0.025
Cytochrome *d*	0.034	0.029	0.014	0.071
Q-8	0.04	< 0.01	2.35	2.26
DMK-8	< 0.01	1.04	< 0.01	0.37
MK-8	< 0.01	0.30	< 0.01	< 0.01

ubiA$^+$ but the membranes contained less than 2 % of the wild-type level of Q-8 and no detectable DMK-8 or MK-8. In all cases the content of FAD, FMN and cytochromes *b*, *o* and *d* in the membranes were within the normal range expected (Table II) and did not appear to be influenced greatly by the presence or absence of Q, DMK or MK.

Requirement for quinones in aerobic electron transport

The oxidase activities present in membranes prepared from the quinone mutants were estimated by measuring the rate of O_2 uptake with NADH, succinate, D-lactate or α-glycerophosphate as substrates. Membranes from strain AN387 (*ubi*$^+$ *men*$^+$) showed high levels of oxidase activity with NADH, succinate and α-glycerophosphate and a lower level of activity with D-lactate. For each substrate little or no stimulation of activity was achieved when Q-3 was added. With membranes of strain AN384 (*ubi*$^-$*men*$^-$), however, the activities of the four oxidase systems were extremely low, but could be restored to levels comparable to those of strain AN387 (*ubi*$^+$*men*$^+$) by the addition of Q-3 (Table III). This indicates that electron transfer from each of the four substrates to oxygen is highly dependent on the presence of a quinone and that Q-3, a short chain homologue of Q-8, can efficiently substitute for Q-8. With membranes from strain AN386 (*ubi*$^+$*men*$^-$) each of the four oxidase activities was normal, indicating that MK-8 and DMK-8 are not required providing Q-8 is present.

Comparison of the results obtained for membranes of strain AN385 (*ubi*$^-$ *men*$^+$) with those of strain AN384 (*ubi*$^-$*men*$^-$) shows that, in the absence of Q-8, the menaquinones (MK-8 and DMK-8) promote full activity for α-glycerophosphate oxidase, approximately half of the Q-3-stimulated activity for D-lactate oxidase, but virtually no activity in the NADH and succinate oxidase systems. MK-1 was found to replace DMK/MK-8 in the oxidation of α-glycerophosphate but not D-lactate (Table III). Although Q-3 and MK-1 can substitute in electron transport for Q-8 and MK-8, respectively, they were found to be about 3-fold less active than the endogenous quinones when tested at equivalent concentrations. This apparent difference in activity is difficult to interpret however, since the short chain quinones were added exogenously in ethanolic solution.

TABLE III

QUINONE REQUIREMENTS FOR OXIDASE SYSTEMS

Rates are expressed as a percentage of the maximally stimulated rates with Q-3 added and are the average of several experiments. The figures in parentheses are the absolute rates in ng atoms O/min per mg protein.

Membrane	Addition*	Rate of oxygen uptake			
		NADH	D-Lactate	Succinate	α-Glycerophosphate
AN387	Q-3	100 (425)	100 (37)	100 (244)	100 (306)
(*ubi*$^+$*men*$^+$)	MK-1	88	90	80	98
	Nil	88	83	90	88
AN384	Q-3	100 (853)	100 (185)	100 (206)	100 (140)
(*ubiA*$^-$*menA*$^-$)	MK-1	12	12	6	81
	Nil	3	4	4	9
AN386	Q-3	100 (502)	100 (79)	100 (395)	100 (172)
(*ubi*$^+$*menA*$^-$)	MK-1	100	80	100	100
	Nil	100	80	100	80
AN385	Q-3	100 (692)	100 (299)	100 (273)	100 (208)
(*ubiA*$^-$*men*$^+$)	MK-1	–	–	–	100
	Nil	13	49	15	90

* Q-3 and MK-1 were added in ethanol to give a final concentration of 27 nmol/mg protein.

TABLE IV

P/O RATIOS FOR AEROBIC ELECTRON TRANSPORT WITH DIFFERENT QUINONES

Additions were added at the following concentrations: Q-3, 27; MK-1, 27; and CCCP, 0.8 nmol/mg protein, respectively.

Membranes from	Natural quinone present	Substrate	Addition	P/O
AN387	Q-8 and DMK/MK 8	NADH	–	0.15
		D-lactate	–	0.12
		succinate	–	0.13
		α-glycerophosphate	–	0.15
AN386	Q-8	NADH	–	0.07
		D-lactate	–	0.10
		succinate	–	0.10
		α-glycerophosphate	–	0.11
AN385	DMK/MK-8	α-glycerophosphate	–	0.09
AN384	Nil	NADH	Q-3	0.05
		D-lactate	Q-3	0.07
		succinate	Q-3	0.08
		α-glycerophosphate	Q-3	0.08
		α-glycerophosphate	MK-1	0.07
		α-glycerophosphate	MK-1 plus CCCP	0.01

P/O ratios were measured for membranes from each of the mutant strains in order to determine whether or not electron transport supported by Q-8, DMK/MK-8 and the short chain homologues Q-3 and MK-1 is coupled to phosphorylation. Table IV shows that the P/O ratios were of similar magnitude irrespective of the quinone present. This indicates that electron transport with Q-8, DMK/MK-8, Q-3 or MK-1 is predominantly via the normal phosphorylating pathway. The P/O ratios were low (generally about 0.1) as expected for this type of membrane preparation. Furthermore the P/O ratios for NADH were no higher than those for succinate suggesting that phosphorylation at site 2 only was being measured.

Pool function of ubiquinone in aerobic electron transport

In order to investigate the possibility that quinones behave as a common pool in the electron transport chain rather than as separated components of each oxidase system we determined whether or not all of the ubiquinone could be reduced by any one substrate. For this purpose we used membranes from a *ubi*$^+$*men*$^-$ strain.

TABLE V

PERCENTAGE REDUCTION OF UBIQUINONE IN MEMBRANES FROM STRAIN AN386 (*ubi*$^+$*menA*$^-$)

The method for the estimation of reduced Q-8 is described in Materials and Methods.

Substrate	Rate of oxygen uptake (ng atoms O/min per mg protein)	Percent Q-8 reduced	
		Steady state	Anaerobic
None	22	12	–
NADH	875	37	81
α-Glycerophosphate	157	17	73
Succinate	280	21	71

It was found (Table V) that about 12 % of the total Q-8 in membranes was in the reduced form without added substrate. With substrates added the proportion of Q-8 in the reduced form in membranes respiring in the aerobic steady state increased to 17 % of the total for α-glycerophosphate, 21 % for succinate and 37 % for NADH. With membrane suspensions which had been allowed to become anaerobic in the presence of excess substrate 70–80 % of the Q-8 was reduced irrespective of which substrate was used (Table V). The Q-8 which remained in the oxidized form in anaerobic membranes could not be reduced by the addition of KCN with each substrate or by the addition of all three substrates together. However, it could be reduced by dithionite. It is possible that this portion of the Q-8 has been disconnected from the electron transport chain by disruption of the membranes during cell breakage; a similar effect has been observed with submitochondrial particles [29].

Concentration of quinones and cytochromes in membranes of mutant strains grown anaerobically with nitrate

Each quinone mutant was grown anaerobically on glucose-minimal medium supplemented with nitrate (see Materials and Methods) and the cells examined for their quinone contents (Table VI). As expected, membranes from strain AN387

TABLE VI

QUINONE LEVELS IN CELLS GROWN AEROBICALLY OR ANAEROBICALLY WITH NITRATE

Cells were grown as described in Materials and Methods.

Strain	Quinone levels (nmol/g wet weight of cells)					
	Aerobically			Anaerobically+nitrate		
	Q	DMK	MK	Q	DMK	MK
AN387 (*ubi*$^+$*men*$^+$)	210	38	5	43	58	59
AN385 (*ubiA*$^-$*men*$^+$)	< 2	120	34	< 2	179	106
AN386 (*ubi*$^+$*menA*$^-$)	235	< 2	< 2	25	< 2	< 2
AN384 (*ubiA*$^-$*menA*$^-$)	4	< 2	< 2	< 2	< 2	< 2

(*ubi*$^+$*men*$^+$) contained Q, DMK and MK, membranes from strain AN385 (*ubi*$^-$*men*$^+$) contained no Q but increased levels of DMK and MK, and membranes from strain AN386 (*ubi*$^+$*men*$^-$) contained a low concentration of Q and no DMK or MK. Strain AN384 (*ubi*$^-$*men*$^-$) was unable to form Q, DMK or MK when grown anaerobically with nitrate. When the quinone concentrations in anaerobic cells were compared with values from strains grown aerobically it was found that the Q concentration fell

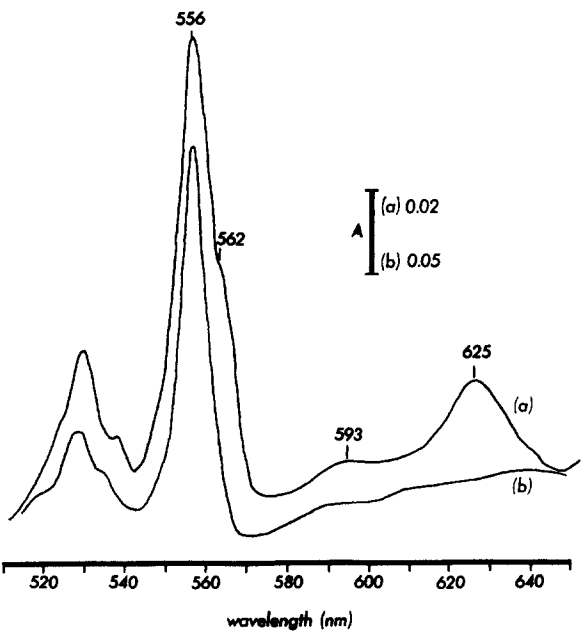

Fig. 1. Dithionite-reduced minus oxidized difference spectra of AN386 membranes at 77 K. Each cuvette (2-mm light path) contained 0.1 ml membranes, 0.4 ml sucrose/TES/Mg^{2+} buffer and 0.5 ml 70% sucrose and spectra were measured using an Aminco Chance spectrophotometer with a spectral bandpass of 1.1 nm. Spectra shown are (a) membranes from aerobically grown cells, $A = 0.2$ full scale; and (b) membranes from cells grown anaerobically with nitrate, $A = 0.5$ full scale.

to 20 % or less of the aerobic concentration while the DMK concentration showed a slight increase and the MK concentration a considerable increase over levels observed for aerobic cells (Table VI).

Cytochrome spectra for respiratory membranes from each of the quinone mutants grown anaerobically with nitrate were measured at low temperature and compared with those for membranes prepared from cells grown aerobically (Fig. 1). In agreement with the results of other workers [15] the peak due to the α band of cytochrome *b* was at 556 nm and was similar in the case of each strain grown anaerobically with nitrate. Based on the absorbance at 556 nm, 2–3-fold more cytochrome *b* was present in anaerobic membranes than in aerobic membranes. No contribution from cytochrome *b*-558 or *b*-562 could be detected in anaerobic membranes.

Requirement for quinones in anaerobic electron transport to nitrate

Membranes prepared from the quinone mutants grown anaerobically were tested for their ability to transport electrons from formate to nitrate. AN387 (*ubi*$^+$ *men*$^+$) membranes were found to have a high level of formate-nitrate reductase activity which was stimulated a further 2- or 3-fold by the addition of either Q-3 or MK-1 (Table VII). In contrast, with AN384 (*ubi*$^-$*men*$^-$) membranes, formate-nitrate reductase was virtually absent unless either Q-3 or MK-1 was added, in which case the activity was reconstituted to levels greater than those in wild-type membranes. Q-3 was found to be more active than MK-1 on a molar basis (the quinones giving full activity at 0.4 and 11 μM, respectively). These results indicate that electron transfer from formate to nitrate is strongly dependent on the presence of a quinone and that either Q or MK can satisfy the quinone requirement. This was confirmed by examining membranes from AN385 (*ubi*$^-$*men*$^+$) and AN386 (*ubi*$^+$*men*$^-$). In both cases the activities were comparable to those for the wild-type strain (Table VII).

Although formate was by far the most active electron donor for the nitrate

TABLE VII

ELECTRON TRANSPORT FROM FORMATE TO NITRATE IN MEMBRANES FROM QUINONE MUTANTS

Membranes from	Addition*	Formate-nitrate reductase (nmol NO_2 produced/min per mg protein)
AN387 (*ubi*$^+$*men*$^+$)	Nil	187
	Q-3	355
	MK-1	493
AN384 (*ubiA*$^-$*menA*$^-$)	Nil	< 10
	Q-3	753
	MK-1	740
AN385 (*ubiA*$^-$*men*$^+$)	Nil	138
	Q-3	237
	MK-1	308
AN386 (*ubi*$^+$*menA*$^-$)	Nil	77
	Q-3	157
	MK-1	277

* Q-3 and MK-1 were added in 3 μl of ethanol to give a final concentration of 48 μM.

TABLE VIII

FUNCTION OF QUINONES IN ELECTRON TRANSPORT FROM VARIOUS SUBSTRATES TO NITRATE USING MEMBRANES FROM AN384 (*ubiA*$^-$*menA*$^-$)

Electron donor	Rate of nitrate reduction (nmol/min per mg protein)		
	No addition	Q-3*	MK-1*
Formate	< 10	753	740
D-Lactate	< 10	159	< 10
α-Glycerophosphate	< 10	57	57
NADH	< 10	59	< 10

* Q-3 and MK-1 were added in 3 μl of ethanol to give a final concentration of 48 μM.

system, other substrates such as D-lactate, α-glycerophosphate and NADH gave much lower rates of nitrate reduction. Using membranes from AN384 (*ubi*$^-$*men*$^-$) it was shown that the reduction of nitrate was highly dependent on the presence of a quinone (Table VIII). Q-3 but not MK-1 was active in electron transport from D-lactate and NADH to nitrate, and either Q-3 or MK-1 could function in electron transport from α-glycerophosphate to nitrate. The maximally stimulated values obtained with AN384 (*ubi*$^-$*men*$^-$) were several-fold higher than the corresponding activities obtained with the parent strain AN387. It should be recalled that with membranes from aerobically grown cells the same quinone preferences were found for the transfer of electrons from these three substrates to oxygen (see above).

Quinones and formate hydrogenlyase activity

The formate hydrogenlyase system of *E. coli* [15] also involves formate dehydrogenase and the requirement of this system for quinones was examined by way of comparison with formate-nitrate reductase. In the case of strain AN387 (*ubi*$^+$*men*$^+$), two separately grown lots of cells gave activities for formate hydrogenlyase of 155 and 231 nmol H_2 evolved/min per mg protein. In similar experiments cells of strain AN384 (*ubi*$^-$*men*$^-$) gave activities of 94 and 112 nmol H_2 evolved/min per mg protein. The fact that cells lacking both ubiquinone and menaquinone have appreciable formate hydrogenlyase activity indicates that a quinone is not an obligatory part of the formate hydrogenlyase system. The lower activity of strain AN384 compared to strain AN387 is probably the result of lack of quinones on general metabolism rather than a specific effect on the formate hydrogenlyase system.

DISCUSSION

The derivation and characterization of a *ubiA*$^-$*menA*$^-$ strain together with the single quinone mutants has provided a useful series of strains for examining the functions of quinones in *E. coli*. The value of a double quinone mutant is particularly evident in cases such as α-glycerophosphate oxidase (Table III) and formate-nitrate reductase (Table VII) where either quinone can function and where examination of a *ubi*$^-$ or *men*$^-$ strain could lead to the conclusion that the oxidation of these substrates was quinone independent. The *ubiA*$^-$*menA*$^-$ strain also provides a "quinone-free"

system which may prove to be useful in determining which processes in electron transport and energy coupling can occur in the absence of quinones. In this regard it is noteworthy that electron transport coupled to phosphorylation can be restored with *ubiA*$^-$*menA*$^-$ membranes by the addition of Q-3 (Table IV).

The experiments described in the present study have confirmed the previous finding [8] that Q-8 is an essential component of NADH oxidase and D-lactate oxidase and, in addition, have defined the role of quinones in succinate oxidase, α-glycerophosphate oxidase and in the nitrate reductase system. In the case of aerobic electron transport each of the four oxidases examined (NADH, D-lactate, α-glycerophosphate and succinate) requires a quinone for activity, however, certain oxidases appear to be more selective than others with respect to the quinone component. Thus NADH oxidase and succinate oxidase require Q-8 for activity and DMK/MK-8 will not substitute for it. With α-glycerophosphate oxidase, however, both Q-8 and DMK/MK-8 promote activity equally well. DMK/MK-8 can also function in D-lactate oxidation but less effectively than Q-8. These results indicate that ubiquinone is the primary quinone under aerobic conditions, being involved in the oxidation of the four substrates tested. Menaquinone can participate in aerobic electron transport but to a more limited extent than ubiquinone, i.e. in the oxidation of α-glycerophosphate and D-lactate. This conclusion is supported by studies on the growth rates and growth yields of the quinone mutants. Thus while the growth yield of strain AN385 (*ubi*$^-$*men*$^+$) was reduced to 30 % of that of the *ubi*$^+$*men*$^+$ strain (AN387), the loss of DMK/MK-8 (*ubi*$^+$*menA*$^-$) strain had no effect on the growth rates or yield. The loss of both quinones results in a strain (*ubiA*$^-$*menA*$^-$) which cannot grow on glucose-minimal medium unless supplemented with casamino acids. It would appear therefore that under aerobic conditions *E. coli* cannot grow solely using glycolysis, but requires some respiratory chain activity. Mutants of *E. coli* (*hemA*$^-$), which are unable to synthesize cytochromes, show similar growth characteristics (unpublished observations).

The fact that the oxidases show specificity with respect to activation by Q-8 or DMK/MK-8 suggests that a quinone site is located between the respective dehydrogenases and the *b* cytochromes of the respiratory chain (see Fig. 2). Thus although DMK/MK-8 can transfer reducing equivalents to the *b* cytochromes (as shown by its ability to function in the oxidation of α-glycerophosphate), it is unable to participate in NADH or succinate oxidation presumably because of an inability to accept electrons from NADH dehydrogenase and succinate dehydrogenase, respectively. This is discussed in more detail in the accompanying paper [30] in terms of the known redox potentials of Q and MK and in relation to the properties of a number of ubiquinone analogues. The findings in the present work do not preclude another site for quinone function in the common portion of the respiratory chain of *E. coli*, as for example postulated in the novel proton motive ubiquinone cycle [31]. The results predict, however, that either Q-8 or DMK/MK-8 would be capable of functioning at such a site.

The concept that the quinones act as acceptors for a variety of different dehydrogenases raises the question of whether or not they behave as a true pool as postulated for ubiquinone in mitochondria [5]. In the present work it was shown that each of the four substrates reduced most of the ubiquinone when membranes were allowed to become anaerobic in the presence of excess substrate. This result is consistent with

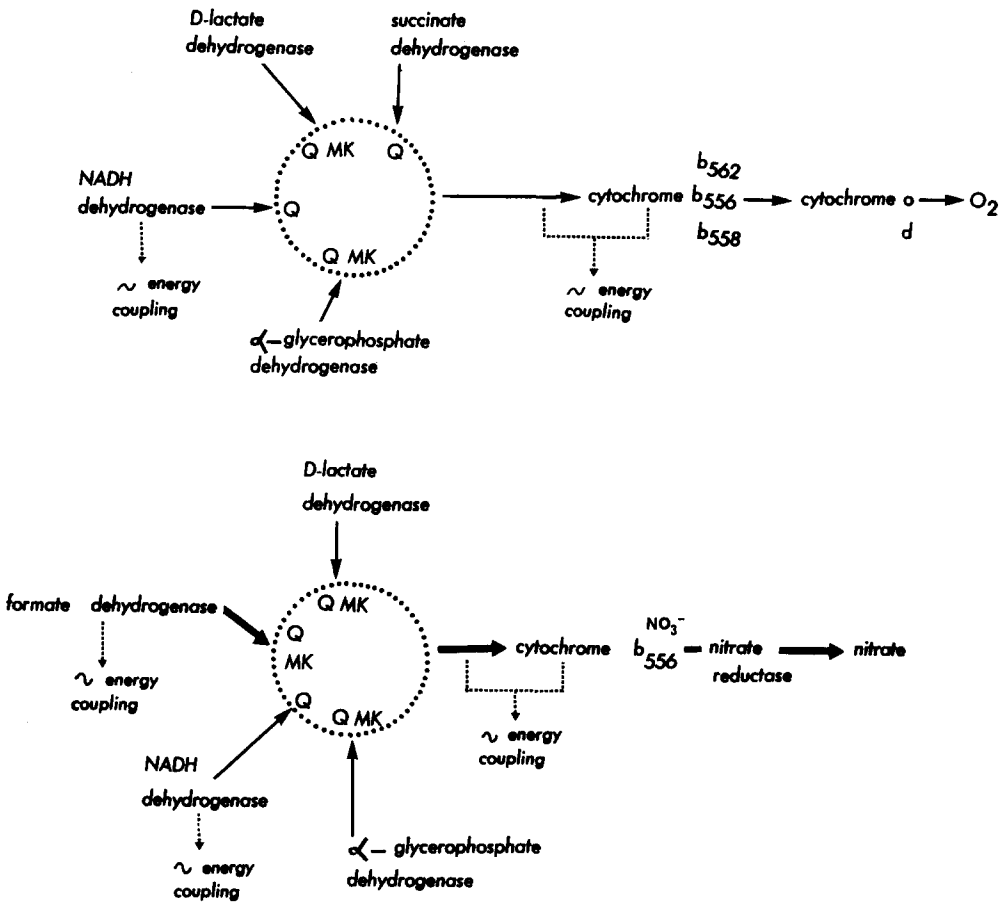

Fig. 2. Hypothetical schemes showing the role of quinones in electron transport to oxygen and nitrate in *E. coli* together with possible sites of energy coupling.

the idea of a pool of ubiquinone common to the dehydrogenases associated with the aerobic respiratory chain.

Quinones are also obligate redox carriers for the nitrate reductase system of *E. coli* (Table VII) where presumably they also serve to link the dehydrogenases to the cytochrome *b*-556 (Fig. 2). It is interesting that although the menaquinones are the major quinones in cells grown under anaerobic conditions, ubiquinone alone can support normal rates of electron transport to nitrate from formate, D-lactate, α-glycerophosphate and NADH. In wild-type cells presumably both quinones participate in electron transport to nitrate according to the quinone specificities of the dehydrogenases. The data of Table VI indicate that DMK-8 is the predominant naphthoquinone under aerobic conditions and that there is a noticeable increase in MK-8 synthesis when cells are grown anaerobically with nitrate. In separate experiments, the DMK-8 produced by *E. coli* K12 has been isolated and its identity confirmed (Young, I. G., unpublished data). The significance of the production of the two naphthoquinones and the changes in their levels is not clear. It is possible that there

are differences in the relative ability of these two quinones to function in the various electron transport systems but it was not possible to test this with the mutants used in this study.

Apart from the nitrate reductase system, *E. coli* also can utilize fumarate anaerobically as an electron acceptor using the fumarate reductase system. Evidence has been obtained using quinone mutants of *E. coli* that menaquinone is involved in linking dihydroorotate dehydrogenase to fumarate reductase [27]. Recently, Singh and Bragg [32] concluded that menaquinone is also involved in α-glycerophosphate-dependent and NADH-dependent reduction of fumarate in *E. coli*.

If one accepts the interpretation of the present work regarding the site of quinone function, then the general picture which emerges is that the quinones are used in *E. coli* as mobile redox carriers to link the dehydrogenases with the three alternative terminal electron transport systems to oxygen, nitrate or fumarate. Energy is conserved in each of the three systems and can be used to drive active transport or synthesize ATP [30, 32–36]. Several schemes have been put forward describing how quinones might function between the dehydrogenases and the terminal electron transport systems in terms of charge separation and proton movements [37–39].

The differential roles of ubiquinone and menaquinone have also been studied in *Proteus rettgeri* by Kröger et al. [6] using extraction-reactivation methods and by following the degree of reduction of the two quinones. Ubiquinone was shown to be involved primarily in aerobic electron transport from succinate, NADH and formate and menaquinone in electron transport to fumarate from NADH and formate. As in *E. coli*, Q was formed preferentially under aerobic and MK under anaerobic conditions. Thus there is a degree of similarity in the differential roles of Q and MK in these two organisms.

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