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The Function of Ubiquinone in *Escherichia coli*

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1. The function of ubiquinone in *Escherichia coli* was studied by using whole cells and membrane preparations of normal *E. coli* and of a mutant lacking ubiquinone. 2. The mutant lacking ubiquinone, strain AN 59 (*Ubi*$^-$), when grown under aerobic conditions, gave an anaerobic type of growth yield and produced large quantities of lactic acid, indicating that ubiquinone plays a vital role in electron transport. 3. NADH and lactate oxidase activities in membranes from strain AN 59 (*Ubi*$^-$) were greatly impaired and activity was restored by the addition of ubiquinone (Q-1). 4. Comparison of the percentage reduction of flavin, cytochrome b_1 and cytochrome a_2 in the aerobic steady state in membranes from the normal strain (AN 62) and strain AN 59 (*Ubi*$^-$) and the effect of respiratory inhibitors on these percentages in membranes from strain AN 62 suggest that ubiquinone functions at more than one site in the electron-transport chain. 5. Membranes from strain AN 62, in the absence of substrate, showed an electron-spin-resonance signal attributed to ubisemiquinone. The amount of reduced ubiquinone (50%) found after rapid solvent extraction is consistent with the existence of ubiquinone in membranes as a stabilized ubisemiquinone. 6. The effects of piericidin A on membranes from strain AN 62 suggest that this inhibitor acts at the ubiquinone sites: thus inhibition of electron transport is reversed by ubiquinone (Q-1); the aerobic steady-state oxidation–reduction levels of flavins and cytochrome b_1 in the presence of the inhibitor are raised to values approximating those found in the membranes of strain AN 59 (*Ubi*$^-$); the inhibitor rapidly eliminates the electron-spin-resonance signal attributed to ubisemiquinone and allows slow oxidation of endogenous ubiquinol in the absence of substrate and prevents reduction of ubiquinone in the presence of substrate. It is concluded that piericidin A separates ubiquinone from the remainder of the electron-transport chain. 7. A scheme is proposed in which ubisemiquinone, complexed to an electron carrier, functions in at least two positions in the electron-transport sequence.

The function of ubiquinone has been the subject of intensive research since Crane, Hatefi, Lester & Widmer (1957) demonstrated that heptane extraction of ox heart mitochondria caused the loss of succinate oxidase activity. This activity could be restored by the addition of cytochrome *c* or a compound ('Q-275') present in the heptane extract and later named coenzyme Q (Lester, Crane & Hatefi, 1958) or ubiquinone (Morton, Wilson, Lowe & Leat, 1957). Szarkowska (1966) extracted freeze-dried ox heart mitochondria with pentane and demonstrated a loss of NADH oxidase activity that could be reversed by the addition of ubiquinone (Q-10) plus mitochondrial phospholipids. Ernster, Lee, Norling & Persson (1969) used the freeze-drying method of Szarkowska and examined the reduction of cytochromes *b*, c_1 and *a* by NADH and succinate in submitochondrial particles. They concluded that the site of inactivation caused by the extraction of ubiquinone (Q-10) occurred in the region between the NADH dehydrogenase or succinate dehydrogenase and cytochrome *b*.

Fragmentation of mitochondria or submitochondrial particles with appropriate reagents separates the respiratory chain into four segments or complexes (Green & Wharton, 1963). Studies of the interaction of the complexes with ubiquinone (Q-2) provided evidence that ubiquinone functions between the flavoproteins and the cytochromes (see Green & Brierley, 1965).

Redfearn & Pumphrey (1960) measured rates of reduction of endogenous ubiquinone by succinate

or NADH in mitochondrial fragments by a chemical extraction method. They found that the rates of reduction of ubiquinone were less than the overall oxidase rates, an observation confirmed by Chance & Redfearn (1961) using a direct spectrophotometric method. The conclusion from these observations was that ubiquinone was not involved directly in the pathway of electron transfer. Storey & Chance (1967), having found agreement between the spectrophotometric and extraction methods, also concluded that ubiquinone is not a component of the NADH oxidase system in the electron-transport particles prepared by alkaline treatment of ox heart mitochondria.

Klingenberg & Kröger (1966), using modified extraction and spectrophotometric techniques with sonicated mitochondrial particles, obtained kinetic data that were consistent with a function for ubiquinone on the main electron-transport pathway. Kröger & Klingenberg (1967) further concluded that ubiquinone functions between the flavoproteins and the cytochromes on the basis of changes in percentage reduction of the various electron carriers during steady-state transitions in whole mitochondria.

Bacterial membranes, unlike mitochondria, may contain ubiquinone or vitamin K_2, or both (Bishop, Pandya & King, 1962). Most of the Gram-positive organisms examined contain only vitamin K, whereas the Gram-negative organisms generally contain only ubiquinone. A few micro-organisms, including *Escherichia coli*, form both ubiquinone and vitamin K.

Azotobacter vinelandii is representative of the group containing only ubiquinone, and Swank & Burris (1969) have restored the NADH oxidase activity of freeze-dried pentane-extracted membranes from this organism by adding back ubiquinone (Q-8). The function of ubiquinone in membranes from *E. coli* has been studied by Kashket & Brodie (1963) using irradiation by light to deplete the membranes of quinones. Examination of the reactivation of NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase by the addition of either of the two quinones formed by *E. coli* led to the conclusion that vitamin K was involved in the NADH oxidase system and that ubiquinone was involved in the succinate oxidase system.

Bacterial mutants have been used only to a limited extent in the study of ubiquinone function (Jones, 1967; Cox, Snoswell & Gibson, 1968). Strains of *E. coli* containing mutations affecting the pathway of ubiquinone biosynthesis have recently been characterized both biochemically and genetically (Cox, Young, McCann & Gibson, 1969). One of these strains has now been used in the present more extensive study of ubiquinone function in *E. coli* K12.

MATERIALS AND METHODS

Chemicals. Piericidin A was kindly provided by Professor S. Tamura, Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan. Ubiquinone (Q-1) and vitamin K (MK-1) were kindly provided by Dr O. Isler of F. Hoffmann–La Roche and Co., Basle, Switzerland. Chemicals generally were of the highest purity available commercially and were not further purified.

Organisms. The strains of *E. coli* K12 used were AB2154, AN59 and AN62; these strains have been described previously (Cox *et al.* 1969). Strain AN59, the mutant lacking ubiquinone, is referred to throughout this paper as strain AN59 (*Ubi*$^-$).

Media and growth of organisms. The minimal medium used (medium 56) was that described by Monod, Cohen-Bazire & Cohn (1951). To the sterilized mineral salts base were added L-leucine, L-threonine and L-methionine each at a final concentration of 0.2mM and thiamin at a final concentration of 0.02μM. Glucose was added as a sterile solution either in excess at a final concentration of 30mM or at limiting concentrations as indicated.

For experiments on growth yields cells were grown at 37°C in 10ml volumes in 125ml conical flasks with a Klett–Summerson tube as a side arm. Limiting concentrations of glucose were used and growth was considered complete when two successive readings, taken at 30min intervals on the Klett–Summerson colorimeter, were similar. The flasks were aerated by shaking at 250rev./min in a New Brunswick Metabolyte shaker-bath. For anaerobic growth conditions the flasks were gassed with nitrogen for 1h while shaking. The medium was supplemented for the anaerobic experiments with $NaHCO_3$ at a final concentration of 25mM and, where indicated, KNO_3 at a final concentration of 0.1%.

For the preparation of cell extracts, organisms were grown at 37°C in 14-litre New Brunswick fermenters with aeration at 10l/min and stirring at 750rev./min. Strain AN59 (*Ubi*$^-$) had a tendency to revert, and to prepare inocula the stock culture was plated out on glucose-nutrient agar plates. If there were no revertant colonies (see Cox *et al.* 1969) after incubation overnight at 37°C the growth from three plates was emulsified and added to a 1-litre culture of the supplemented medium 56. After incubation with shaking at 37°C overnight this culture was used to inoculate 10 litres of medium in the 14-litre fermenter.

Preparation and fractionation of cell extracts. Cells were grown as described above and the cultures were harvested early in growth (0.2–0.4mg dry wt./ml). The cells were washed once in cold 0.1M-potassium phosphate buffer, pH7.0, and resuspended in fresh buffer (1ml of buffer/0.5g wet wt. of cells), and a cell extract was prepared by passing the suspension through a Sorvall Ribi Cell Fractionator at 20000lb/in^2. The membrane fraction was prepared from the cell extract by adding solid $(NH_4)_2SO_4$ slowly to give 30% saturation and stirring for a further 30min. The precipitate was collected after centrifugation at 25000*g* for 30min and resuspended in 1ml of 0.1M-phosphate buffer, pH7.0, for each original 1g wet wt. of cells. For the experiments described in Table 2 the precipitate was resuspended in 2ml of buffer per original 1g wet wt. of cells. For electron-spin-resonance measure-

ments the precipitate was resuspended in 0.2ml of buffer for each original 1g wet wt. of cells. All operations on the harvested cells and cell fractions were carried out at 0–4°C. Proteins were estimated with Folin's phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin (Fraction V, Sigma Chemical Co., St Louis, Mo., U.S.A.) as standard.

Determinations of glucose and lactic acid. Glucose was determined by the hexokinase–glucose 6-phosphate dehydrogenase method as described by Slein (1965). Lactic acid was determined colorimetrically after oxidation to acetaldehyde as described by Barker (1957).

Determination of fermentation products. The determinations of acetic acid, lactic acid and ethanol were kindly carried out by Dr H. Doelle of the University of Queensland using a g.l.c. technique (Doelle, 1969).

Measurement of oxygen uptake. Oxygen uptakes at 25°C were measured polarographically with a Titron oxygen electrode (Titron Instruments, Melbourne, Vic., Australia) modified as described by Snoswell (1966). The reaction mixture contained (final concentrations) 15mM-sodium–potassium phosphate buffer, pH7.4, 1.9mM-$MgCl_2$, 1–5mg of protein, 2mM-substrate (except for NADH, which was 1.2mM) and, where indicated, 0.6mM-NAD^+ and 0.6mM-$NADP^+$ in a final volume of 2.5ml. Buffer solutions were calibrated for oxygen content by the method of Chappell (1964).

Determinations of quinones. The ubiquinone and vitamin K contents of the membrane fraction were determined as described previously (Cox *et al.* 1968). The maximum value quoted for the ubiquinone content of strain AN59 (*Ubi*$^-$) was determined after the extraction of large quantities of whole cells during the isolation of 2-octaprenylphenol (Cox *et al.* 1969).

Difference spectra. Difference spectra were recorded in a Cary 14R spectrophotometer with a scattered transmission accessory and a 0–0.1A slide-wire. Membrane preparations were diluted to a concentration of approx. 10mg of protein/ml and the differences between $Na_2S_2O_4$-reduced and oxygenated samples were recorded. The wavelength pairs and molar extinction coefficients were those employed by Jones & Redfearn (1966): cytochrome b_1, $\Delta E_{560}-\Delta E_{575}$ (ϵ_M17500); cytochrome a_2, $\Delta E_{630}-\Delta E_{615}$ (ϵ_M8500); total flavoprotein, $\Delta E_{465}-\Delta E_{510}$ (ϵ_M11000). The determination of flavoprotein by this method may be subject to error due to absorption by non-haem iron. Cytochrome *o* concentrations were determined from the $Na_2S_2O_4$-reduced+CO minus $Na_2S_2O_4$-reduced difference spectra, by using the molar extinction coefficient calculated by Taber & Morrison (1964); $\Delta E_{415}-\Delta E_{430}$ (ϵ_M80000). Cytochrome a_1 was not present in sufficient quantity for accurate determination.

Determinations of steady-state oxidation–reduction levels. The reduction kinetics of the individual cytochrome and flavin components in the membrane fractions were determined with an Aminco–Chance dual-wavelength spectrophotometer. A slit width of 0.25mm (dispersion, 5.5nm/mm) was used. The following wavelength pairs were employed: cytochrome b_1, 560 and 570nm; cytochrome a_2, 630 and 615nm; flavoprotein, 475 and 495nm. The measurements were carried out at 25°C in the 5% transmission range. The reaction mixture contained (final concentrations) approx. 5mg of membrane protein, 1.6mM-$MgCl_2$, 28mM-sodium–potassium phosphate buffer, pH7.4, in a final volume of 3ml. The mixture was well aerated by shaking and the reference and sample light-beams were balanced. The reaction was initiated by stirring in 30μl of substrate (5μmol of NADH or 15μmol of D-lactate). The reduction of a component in the aerobic steady state was expressed as a percentage of its final reduction after the exhaustion of all of the oxygen. Measurements could be repeated, after shaking the cuvette, until the substrate was exhausted. At the end of each experiment $Na_2S_2O_4$ was added to check total reduction and the results indicated that 80–90% of each component had been reduced by substrate. In the presence of cyanide (0.8mM) the cytochrome a_2 formed a complex of altered spectrum and therefore these steady-state measurements were only approximate. In cases where the percentage reduction of a component in the steady state was still rising when the system went anaerobic, the steady-state values determined after reshaking were taken as being more accurate. Inhibitors were added several minutes before the addition of substrate, with the exception of cyanide, which was incubated for 10min with undiluted membranes before the addition of buffer.

The degree of reduction of ubiquinone in the aerobic steady state was measured after determination of total ubiquinone and oxidized ubiquinone by the method of Hoffmann, Kunz, Schmid & Siess (1964). Incubations were carried out, with stirring, in volumes of 1.5ml at 25°C and the reaction was stopped by the addition of 5ml of light petroleum (b.p. 40–60°)–methanol (2:3, v/v) as described by Kröger & Klingenberg (1966).

Measurement of electron-spin-resonance spectra. The *X*-band spectra were recorded as first-derivative traces with a Varian V-4501 spectrometer. To an electron-spin-resonance tube was added 0.1ml of 15mM-sodium–potassium phosphate buffer, pH7.4, containing 1.9mM-$MgCl_2$ and, where indicated, 10μl of inhibitor. The membrane preparation (0.1ml containing 15mg of protein) was then added and mixed with a syringe. Spectra were recorded after freezing the samples in liquid nitrogen. Mixing and freezing the contents of the tube took 30s. The concentrations of unpaired spins were calculated by double integration by comparison with a standard Varian dilute pitch sample.

The electron-spin-resonance recording conditions were as follows: temperature, 77°K; microwave frequency, 9.05GHz; modulation frequency, 100kHz; modulation level, 3.78G peak-to-peak; power, 61mW; integrating time-constant, 1s; gain setting, ×1000; scanning rate, 500G/10min. A Fieldial was used for the direct calibration of field strength.

RESULTS

A ubiquinone-deficient strain of *E. coli* K12 (AB3285) has been used in a study of ubiquinone function in malate oxidation (Cox *et al.* 1968). This strain was derived by conjugation involving a male parent carrying a number of uncharacterized mutations, some of which affected growth on succinate (G. B. Cox, unpublished work), and was subject to the objection that an additional mutation modifying the effect of the ubiquinone deficiency may have been transferred. This objection has been

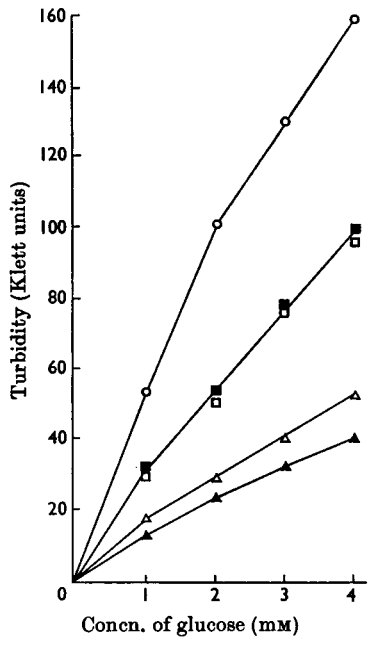


Fig. 1. Growth yields (turbidity) of strains AB2154, AN62 and AN59 (*Ubi*$^-$) grown on limiting concentrations of glucose under various conditions. Cultures were aerated by shaking and anaerobic cultures were incubated under nitrogen as described in the Materials and Methods section. ○, Strain AB2154 or strain AN62, aerobic; △, strain AN62, anaerobic; □, strain AN62, anaerobic with nitrate; ▲, strain AN59 (*Ubi*$^-$), aerobic or anaerobic; ■, strain AN59 (*Ubi*$^-$), anaerobic with nitrate.

minimized by the use of a co-transduction system for the preparation of the ubiquinone-deficient strain AN59 (*Ubi*$^-$) from strain AB2154 (Cox *et al.* 1969). Strain AN62 is a spontaneous revertant from strain AN59 (*Ubi*$^-$) and is able to form ubiquinone (Cox *et al.* 1969). The mutation in strain AN59 (*Ubi*$^-$) causes the accumulation in the cells of the intermediate 2-octaprenylphenol. Although this compound cannot be involved in functions requiring a quinone, it may assist in maintaining membrane structure in the absence of ubiquinone.

Growth yields. The growth yields obtained for strains AB2154, AN59 (*Ubi*$^-$) and AN62 from limiting concentrations of glucose under various conditions of growth were estimated as turbidity in a Klett–Summerson colorimeter as described in the Materials and Methods section. Strains AB2154 and AN62, as expected, gave similar growth yields under aerobic conditions (Fig. 1). However, strain AN59 (*Ubi*$^-$) under aerobic conditions gave a similar low growth yield to those obtained from both strain AN59 (*Ubi*$^-$) and strain AN62 under anaerobic conditions (Fig. 1). These results suggest that ubiquinone is essential for electron transport or for phosphorylation coupled to electron transport. The energy obtained from respiration with nitrate as terminal electron acceptor is apparently unaffected by the absence of ubiquinone, as the growth yields of both strains AN59 (*Ubi*$^-$) and AN62 are similar under anaerobic conditions in the presence of nitrate.

The metabolism of strains AN59 (*Ubi*$^-$) and AN62 under aerobic conditions was examined in more detail. Cultures were sampled at various stages of growth and the samples assayed for glucose and lactate. The results (Fig. 2) confirm the comparatively inefficient conversion of glucose into cell mass in strain AN59 (*Ubi*$^-$). The lack of ubiquinone also causes the accumulation of D-lactate in the culture medium, further suggesting that ubiquinone-deficient cells derive their energy from glycolysis even when grown under aerobic conditions.

The normal products of glucose fermentation by *E. coli* in media of slightly acid pH are lactate, acetate, ethanol, carbon dioxide and hydrogen (Wood, 1961). Lactate, acetate and ethanol were determined in supernatants from cultures of strains AN59 (*Ubi*$^-$) and AN62, grown aerobically on excess of glucose. Strain AN59 (*Ubi*$^-$) formed high concentrations of lactate compared with strain AN62, but normal aerobic concentrations of the other fermentation products, acetate and ethanol (Table 1).

Oxidase systems in cell-free preparations from strain AN62. The revertant strain, AN62, was grown under conditions of good aeration with glucose as carbon source. Cell extracts were prepared by using a Sorvall–Ribi cell fractionator and examined for the presence of various oxidase systems by using an oxygen electrode. The NADH oxidase and D-lactate oxidase systems were quantitatively the most significant (Table 2). The pyruvate oxidase and α-oxoglutarate oxidase systems required the addition of NAD^+ and probably the oxidase activity was due to the presence of the NADH oxidase. The isocitrate oxidase activity required the addition of both NAD^+ and $NADP^+$ and presumably both transhydrogenase and NADH oxidase were involved. There was little or no activity obtained with any of the four substrates succinate, dihydro-orotate, α-glycerophosphate or formate.

The addition of ammonium sulphate (30% saturation) to the cell extract precipitated the NADH oxidase, malate oxidase and lactate oxidase systems without any significant loss of total activity (Table 2); this fraction is referred to below as the membrane fraction. The lactate oxidase and

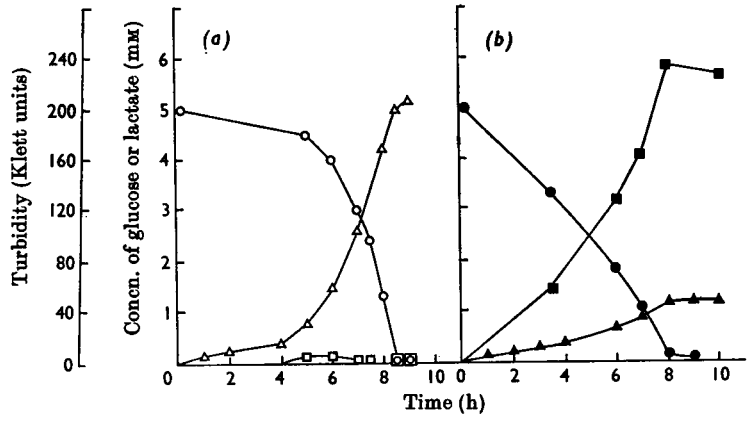


Fig. 2. Aerobic metabolism of glucose by (*a*) strain AN62 and (*b*) strain AN59 (*Ubi*⁻). △ and ▲, Growth; ○ and ●, glucose concentration; □ and ■, lactate concentration. The times were measured from the start of observable growth. Conditions for aerobic growth were as indicated in Fig. 1.

Table 1. *Products of glucose metabolism formed by strains AN62 and AN59 (Ubi⁻) grown under aerobic conditions*

Determinations were made on supernatants from cultures of cells grown for the preparation of membranes as described in the Materials and Methods section. Products were determined by g.l.c. (Doelle, 1969).

Strain	Product (mg/l)		
	Ethanol	Acetate	Lactate
AN62	11	48	86
AN59 (*Ubi*⁻)	7	17	2500

malate oxidase activities were not stimulated by the addition of NAD^+. The loss of pyruvate oxidase and α-oxoglutarate oxidase activities is presumably due to the separation of the primary dehydrogenase and the NADH oxidase. The supernatant fraction after the ammonium sulphate precipitation did not have any detectable oxidase activity with the substrates tested, although a comparatively low NADH oxidase activity was detected on the addition of FAD.

The membrane fraction from strain AN62 was used to test the effect of some known electron-transport inhibitors on the NADH oxidase and lactate oxidase systems. Piericidin A and HQNO* were the most potent inhibitors of both oxidase systems (Table 3) and the inhibition by these compounds was reversed by the addition of ubiquinone (Q-1). Dicoumarol and sodium cyanide inhibited both systems, but sodium Amytal inhibited the lactate oxidase without having any effect on the NADH oxidase. It was not possible to demonstrate any inhibition of the NADH oxidase or lactate oxidase systems by rotenone or antimycin A.

*Abbreviation: HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.

Table 2. *Oxidase systems in cell extract and the membrane fraction from strain AN62*

Rates of oxygen uptake were measured with an oxygen electrode in a volume of 2.5ml at 25°C. The reaction mixture contained 15mM-sodium–potassium phosphate buffer, pH7.4, 1.9mM-$MgCl_2$ and about 4mg of protein for the cell extract or about 1.5mg of protein for the membrane preparation. The membrane fraction was prepared by $(NH_4)_2SO_4$ precipitation of the cell extract and resuspended to the original volume of cell extract as described in the Materials and Methods section. Substrates were added in a volume of 10μl to give a final concentration of 2mM except for NADH (1.2mM). NAD^+ and $NADP^+$ were added to give a final concentration of 0.6mM. Values given are the averages of several determinations on at least five different preparations of membranes and the endogenous oxygen uptakes (about 10ng-atoms of O/min per 100μl for cell extracts and about 2ng-atoms of O/min per 100μl for membrane fractions) have been subtracted.

Addition to buffer	O_2 uptake by preparation (ng-atoms of O/min per 100μl)	
	Cell extract	Membrane fraction
NADH	263	281
D-Lactate	80	76
DL-Malate	28	23
Pyruvate+NAD^+	90	0
α-Oxoglutarate+NAD^+	43	0
Isocitrate+NAD^++$NADP^+$	23	<5

Table 3. *Effects of inhibitors on NADH oxidase and lactate oxidase systems in membranes from strain AN62*

Oxygen uptakes were measured as described in Table 2. The inhibitors were added to the reaction mixture 2 min before the addition of substrate, except for cyanide, which was incubated with membranes at 25°C for 5 min before addition to the electrode vessel. Ubiquinone was added after the addition of substrate.

Inhibitor and final concn. (μM)	Final concn. of ubiquinone (Q-1) (μM)	Inhibition (%) of the oxidation of	
		NADH	D-Lactate
Piericidin A (8)	0	69	32
Piericidin A (24)	0	86	64
Piericidin A (40)	0	92	—
Piericidin A (8)	32	46	—
Piericidin A (8)	64	33	12
HQNO (16)	0	57	—
HQNO (48)	0	82	—
HQNO (80)	0	88	71
HQNO (16)	32	40	—
HQNO (16)	64	28	—
NaCN (200)	0	50	63
NaCN (506)	0	84	—
Dicoumarol (200)	0	49	—
Dicoumarol (400)	0	75	67
Dicoumarol (400)	32	75	—
Sodium Amytal (2800)	0	0	40
Sodium Amytal (4000)	0	0	77

Ubiquinone requirement for oxidase activity. The NADH oxidase, lactate oxidase and malate oxidase systems present in cell extracts of strain AN59 (*Ubi*$^-$) were similarly precipitated by 30% ammonium sulphate saturation. The NADH oxidase, lactate oxidase and malate oxidase activities were all markedly decreased in the membrane fraction from strain AN59 (*Ubi*$^-$) as compared with those in membranes from strain AN62 (Table 4). There was, however, some activity retained and this activity was stimulated to a value greater than that found in strain AN62 by the addition of ubiquinone (Q-1). The addition of vitamin K (MK-1) had a relatively slight effect and the ubiquinone isoprenologues Q-6 and Q-8 were inactive.

Concentrations of membrane components in strains AN59 (Ubi$^-$) *and AN62.* The concentrations of the flavins and cytochromes in the membrane fractions of strain AN59 (*Ubi*$^-$) and strain AN62 were determined from the reduced minus oxidized difference spectra. The cytochrome b_1 concentrations were the same in each strain (Table 5) and, although the flavin and cytochrome a_2 concentrations were higher and the cytochrome *o* concentration was lower in strain AN59 (*Ubi*$^-$), the differences are small considering the different metabolism of the two strains. The ubiquinone and vitamin K contents were determined after extraction and purification as described in the Materials and Methods section. Ubiquinone was present in strain AN62 at a concentration 25 times that of cytochrome b_1. The vitamin K concentration was derepressed fourfold in the absence of ubiquinone.

Table 4. *Comparison of the oxidase systems in strain AN62 and AN59* (Ubi$^-$) *and the effects of ubiquinone* (*Q*-1) *and vitamin K* (*MK*-1)

Oxygen uptakes were measured as described in Table 2. Ubiquinone (Q-1) or vitamin K (MK-1) were added after the addition of substrate. Values represent the averages of experiments with at least ten different preparations of membranes.

Substrate	Quinone added and final concn. (μM)	O_2 uptake (ng-atoms/min per mg of protein) by membranes from	
		Strain AN62	Strain AN59 (*Ubi*$^-$)
NADH	—	230	25
NADH	Q-1 (32)	430	560
NADH	Q-1 (48)	430	560
NADH	MK-1 (32)	230	82
NADH	MK-1 (128)	210	72
D-Lactate	—	68	19
D-Lactate	Q-1 (32)	150	210
D-Lactate	MK-1 (32)	77	30
D-Lactate	MK-1 (128)	—	26
DL-Malate	—	31	5
DL-Malate	Q-1 (32)	50	42

Table 5. *Concentrations of some membrane components in strains AN62 and AN59* (Ubi$^-$)

Flavins and cytochromes were determined by direct spectrophotometric examination of suspensions of the membranes. Quinones were first extracted and partially purified before spectrophotometric determination. Details of methods are given in the Materials and Methods section.

Component	Concn. of component (nmol/mg of protein) in	
	Strain AN62	Strain AN59 (*Ubi*$^-$)
Total flavin	0.25	0.39
Cytochrome b_1	0.19	0.19
Cytochrome a_2	0.027	0.047
Cytochrome *o*	0.073	0.04
Cytochrome a_1*	+	+
Ubiquinone	4.7	<0.05
Vitamin K_2	0.67	2.7

* Cytochrome a_1 was present but the quantities were too low for determination.

Percentage reduction of membrane components in the aerobic steady state. During the passage of electrons from substrate to oxygen via the respiratory chain, the oxidation–reduction levels of various components in this steady state may be determined by direct spectrophotometric methods. The values obtained depend on the balance of oxidase and dehydrogenase activities in the preparation. If, however, an inhibitor is added, then the components of the chain on the substrate side of the point of inhibition become more reduced and those on the oxygen side become more oxidized (Chance & Williams, 1956). Ubiquinone deficiency causes 'inhibition' of the various oxidase systems and, in an attempt to localize the point of inhibition, a comparison of the steady state levels of the components in the membrane fractions from strains AN 59 (*Ubi*$^-$) and AN 62 was made.

The percentage reduction in the aerobic steady state of total flavin, cytochrome b_1 and cytochrome a_2 for strains AN 59 (*Ubi*$^-$) and AN 62 with NADH or lactate as substrate may be seen in Table 6 and Fig. 3. The percentage of flavin and cytochrome b_1 reduced in the aerobic steady state was increased in strain AN 59 (*Ubi*$^-$) with either NADH or lactate as substrate when compared with strain AN 62. These results indicate that ubiquinone has a function after cytochrome b_1. The oxidation–reduction levels in strain AN 59 (*Ubi*$^-$) were returned to those in strain AN 62 by the addition of ubiquinone (Q-1). The percentage reduction of cytochrome a_2 in the aerobic steady state was low in membranes prepared from either strain.

The effects of the inhibitors piericidin A, HQNO, sodium cyanide and sodium Amytal on the percentage reduction, in the steady state, of membrane components from strain AN 62 with NADH or lactate as substrate were examined (Table 6 and Fig. 3). Sodium cyanide, at a concentration inhibiting electron transport less than ubiquinone deficiency, caused a greater increase in the percentage of cytochrome b_1 reduced than did ubiquinone

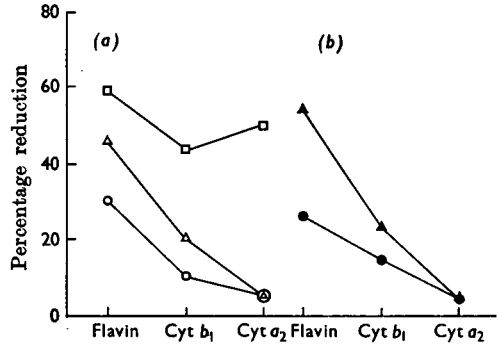


Fig. 3. Percentage reduction of respiratory components in the steady state, with NADH as substrate, in membranes from (*a*) strain AN 62 and (*b*) strain AN 59 (*Ubi*$^-$): the effects of piericidin A or cyanide on strain AN 62 and the effect of added ubiquinone (Q-1) on strain AN 59 (*Ubi*$^-$). Conditions of experiments were as described in Table 6. ○, Strain AN 62; △, strain AN 62 plus piericidin A; □, strain AN 62 plus cyanide; ▲, strain AN 59 (*Ubi*$^-$); ●, strain AN 59 (*Ubi*$^-$) plus ubiquinone (Q-1). Abbreviation: Cyt, cytochrome.

Table 6. *Percentages of flavin, cytochrome* b_1 *and cytochrome* a_2 *reduced in the aerobic steady state in membranes from strains AN* 62 *and AN* 59 (Ubi$^-$)

The steady state oxidation–reduction levels were determined by using a dual-wavelength spectrophotometer as described in the Materials and Methods section. The values for the steady state levels represent the averages of several determinations on at least six different membrane preparations. The times taken for membranes to remove the oxygen are given as an indication of the oxidase rates.

Membrane from	Addition and final concn. (μM)	Approximate time (s) taken to remove dissolved oxygen in the presence of		Steady-state percentage reduction of components in the presence of NADH			Steady-state percentage reduction of components in the presence of Lactate		
		NADH	Lactate	Flavin	Cyto-chrome b_1	Cyto-chrome a_2	Flavin	Cyto-chrome b_1	Cyto-chrome a_2
AN 62	—	7	20	30	10	<5	19	8	<5
AN 62	NaCN (800)	20	80	59	43	>50	27	29	>50
AN 62	Piericidin A (130)	70	70	46	20	<5	30	13	<5
AN 62	HQNO (130)	80	120	42	18	<5	18	12	<5
AN 62	Sodium Amytal (3000)	7	50	30	10	<5	17	2	<5
AN 59	—	60	140	54	23	<5	31	24	<5
AN 59	Ubiquinone (Q-1) (40)	<5	10	26	14	<5	15	7	<5

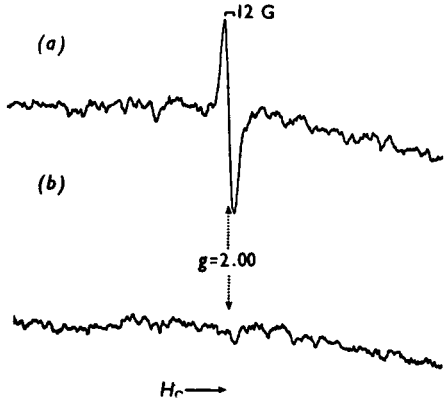


Fig. 4. Effect of inhibitors on the electron-spin-resonance spectrum of membranes from strain AN62 in the absence of added substrate. (*a*) Spectrum from membranes in buffer. A similar spectrum was obtained in the presence of sodium cyanide (12.5mM). (*b*) Spectrum of the same membrane preparation in the presence of either piericidin A (2mM) or HQNO (2mM). The preparation of the membrane samples and the technique used for the preparation of the electron-spin-resonance samples are described in the Materials and Methods section.

deficiency. The effect of piericidin A and HQNO on the steady state oxidation–reduction levels of both flavin and cytochrome b_1 was, however, similar to the effect of ubiquinone deficiency (Fig. 3). Therefore, although ubiquinone deficiency, piericidin A, HQNO and sodium cyanide all inhibit after cytochrome b_1, the effect of sodium cyanide differs from those of the other three in the degree of increase in the percentage reduction of cytochrome b_1 in the steady state (see the Discussion section). Sodium Amytal, which inhibits only the lactate oxidase system, appears to inhibit before cytochrome b_1.

Formation of ubisemiquinone. Electron-spin-resonance studies on membrane fractions from strains AN59 (*Ubi*$^-$) and AN62 indicated the presence of a signal in the strain AN62 preparations attributed to the semiquinone of ubiquinone (Hamilton, Cox, Looney & Gibson, 1970). The signal was present in the absence of substrate, reaching a maximum value of approx. 2% of the total ubiquinone present, and rapidly disappeared after the addition of NADH. The effect of the inhibitors, sodium cyanide, piericidin A and HQNO on the appearance of the signal in membranes from strain AN62 in the absence of substrate are shown in Fig. 4. Although sodium cyanide had little effect on ubisemiquinone formation, the signal was not observed in the presence of piericidin A or HQNO.

Extraction of the ubisemiquinone from membranes by organic solvents would presumably yield a quinol–quinone mixture by a disproportionation reaction. It was therefore decided to examine the percentage of ubiquinone in the reduced form in membrane preparations from strain AN62 by the rapid solvent-extraction method of Kröger & Klingenberg (1967). The percentage of ubiquinone reduced after 2min incubation under aerobic conditions was 50% (Table 7) and this percentage remained constant for at least a further 8min of incubation. However, the addition of piericidin A caused the percentage reduction of ubiquinone to decrease to 38% after 2min of incubation and after a further 8min the ubiquinone was essentially fully oxidized. Addition of the substrate NADH to membranes, whether preincubated for 2, 10 or 20min, caused the percentage of ubiquinone reduced in the aerobic steady state to rise to 55%. If, however, the membranes were preincubated in the presence of piericidin A the addition of the substrate NADH did not change the ubiquinone from its fully oxidized state. This is in marked contrast with the effect of piericidin A on the percentage reduction of flavin and cytochrome b_1 in the aerobic steady state (see above).

Table 7. *Percentage reduction of ubiquinone in the aerobic steady state and the effect of piericidin A*

Membrane preparations (0.2ml) containing about 6mg of protein were incubated with constant shaking at 25°C with 1.3ml of 30mM-sodium–potassium phosphate buffer, pH7.4, containing 7.5mM-$MgCl_2$. The percentage reduction of ubiquinone was measured as described in the Materials and Methods section. Piericidin A, where included, was added (final concn. 107μM) to the buffer before the addition of the membranes.

Time of incubation without substrate (min)	Final concn. of NADH* (mM)	Percentage reduction of ubiquinone in	
		Membranes	Membranes plus piericidin A
2	0	52	38
10	0	51	2
20	0	32	3
20	2.0	55	0

*Incubation was continued for 30s after addition of NADH.

The effect of HQNO on the percentage reduction of ubiquinone was similar to, but not as pronounced as, that of piericidin A. Thus the effect of incubating the membranes with HQNO (107μM) for 10min was to lower the percentage reduction of ubiquinone in the absence of substrate from 51 to 38%.

DISCUSSION

The relationship between growth yield and the amount of substrate utilized in cultures growing

under aerobic or anaerobic conditions is well established (see, for examples, Bauchop & Elsden, 1960; Kormančíková, Kováč & Vidová, 1969). Comparison of the growth yields given by strains AN 62 and AN 59 (*Ubi*$^-$) indicate that phosphorylation coupled to electron transport with oxygen as terminal acceptor does not occur in the absence of ubiquinone. The accumulation of lactic acid by strain AN 59 (*Ubi*$^-$) also suggests that, even under aerobic growth conditions, glycolysis is the main source of energy. However, the metabolism of strain AN 59 (*Ubi*$^-$) has not simply been changed to an anaerobic type of mixed acid fermentation (Wood, 1961), since only relatively small amounts of acetic acid and ethanol are formed. *Escherichia coli*, when grown aerobically on glucose as carbon source, forms an NAD-linked lactate dehydrogenase that functions unidirectionally in producing the D-isomer of lactic acid (Tarmy & Kaplan, 1968). In addition, a flavoprotein-linked membrane-bound D-lactate oxidase is formed that functions in the opposite direction, i.e. formation of pyruvate from D-lactate (Kline & Mahler, 1965). The accumulation of D-lactate in aerobic cultures of strain AN 59 (*Ubi*$^-$) is due not only to the decrease in D-lactate oxidase activity but also to a fourfold increase in the NAD-linked lactate dehydrogenase (P. Stroobant, unpublished work).

A number of *E. coli* K 12 strains grown on glucose as carbon source have been examined for the presence of various oxidase systems; the NADH oxidase and D-lactate oxidase systems were always quantitatively the most important, whereas the presence of other systems, such as the malate oxidase and the succinate oxidase, varied from strain to strain (G. B. Cox, unpublished work). It is clear that ubiquinone is involved in the NADH oxidase, lactate oxidase and malate oxidase systems, since the oxidase activities were much lower in strain AN 59 (*Ubi*$^-$) than in the revertant strain AN 62. Further, the addition of ubiquinone (Q-1) to membranes from AN 59 (*Ubi*$^-$) caused an immediate increase in oxidase activities to values greater than those found in membranes from strain AN 62.

The inhibitions of the NADH oxidase system by dicoumarol and HQNO found in the present work are similar to those found by Bragg & Hou (1967) and Jones (1967), whereas our preparations were somewhat more sensitive to cyanide. The inhibitors piericidin A and HQNO are alike in that they inhibited at low concentrations and both inhibitions were reversed by added ubiquinone (Q-1).

The results of the steady state experiments indicate that, whereas ubiquinone deficiency, piericidin A, HQNO and sodium cyanide all inhibit electron transport after cytochrome b_1, the effect of cyanide differs from those of the other three in the degree of increase in the percentage reduction of cytochrome b_1 in the steady state. Thus, with NADH as substrate, cyanide used at a concentration causing less inhibition of electron transport than ubiquinone deficiency increased the percentage reduction of cytochrome b_1 some fourfold while increasing that of flavin twofold. However, ubiquinone deficiency, and the inhibitors piericidin A and HQNO added at a concentration giving a greater inhibition of electron transport than by cyanide, caused only a twofold increase in the percentage reduction of cytochrome b_1. These observations are consistent with ubiquinone functioning, and piericidin A and HQNO inhibiting, both before and after cytochrome b_1. Jones (1967) has proposed that ubiquinone functions between flavin and cytochrome b_1 in the NADH oxidase system of *E. coli*, although Kashket & Brodie (1963) have suggested that vitamin K rather than ubiquinone functions at this site. Evidence for a quinone functioning after cytochrome b_1 is provided by Krogstad & Howland (1966) working with the succinate oxidase system of *Corynebacterium diphtheriae*, in which the only quinone is vitamin K.

In the lactate oxidase system of strain AN 62 ubiquinone appears to function only after cytochrome b_1, since the percentage reduction of this cytochrome in membranes from strain AN 59 (*Ubi*$^-$) was not markedly different from that in membranes from strain AN 62 in the presence of cyanide. The lactate oxidase system, as distinct from the NADH oxidase system, was inhibited by Amytal, and the percentage reduction of cytochrome b_1 in the aerobic steady state was lower in the presence of Amytal, indicating that it inhibits before cytochrome b_1. However, the percentage reduction of flavin was about the same in the presence or absence of Amytal, and therefore Amytal may inhibit, not immediately before cytochrome b_1, but between two flavoproteins.

The high percentage of ubiquinone in the reduced form in membranes from strain AN 62 in the absence of substrate is in sharp contrast with the other electron carriers examined, all of which are essentially fully oxidized before the addition of substrate. Further, ubiquinone is present in the membranes at a concentration some 25 times that of cytochrome b_1. The values found for the percentage reduction of ubiquinone could possibly arise from disproportionation of ubisemiquinone on extraction into organic solvent. However, quantitative determination, from electron-spin-resonance spectra of the ubisemiquinone present in membranes indicates that about 2% of the ubiquinone was present in the radical form. If the values found for reduced ubiquinone (up to 50%), as measured by extraction, were due to disproportionation then essentially all of the ubiquinone should be in the radical form.

The explanation for this discrepancy is not known. However, electron-spin-resonance measurements were recorded under quite different conditions (e.g. of aeration, temperature and concentration of membranes) from those used in the measurements of the amounts of reduced ubiquinone by the rapid solvent-extraction technique. Also, there are difficulties associated with the quantitative determination of free radicals (Beinert & Palmer, 1965), and there is also the possibility that an appreciable amount of the ubisemiquinone radical is involved in a metal chelate complex (see below) devoid of an electron-spin-resonance signal (cf. Beinert & Hemmerich, 1965).

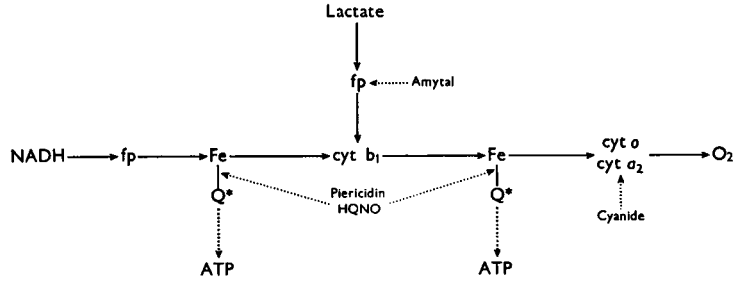
Piericidin A, in the absence of substrate, eliminated the electron-spin-resonance signal and caused the oxidation of reduced ubiquinone (as measured by solvent extraction), although at a lower rate than the elimination of the electron-spin-resonance signal. This inhibitor also prevented a reduction of ubiquinone after the addition of substrate, an observation in marked contrast with its effects in raising the values for the percentage reduction of flavin and cytochrome b_1 in the steady state.

The inhibition by piericidin A of ubiquinone reduction on the addition of substrate has also been found previously (Snoswell & Cox, 1968) with a different strain of *E. coli*. However, in the earlier work ubiquinone was found to be fully oxidized in the absence of substrate, an effect probably due to the use of a different membrane ('small-particle') preparation rather than a difference in strains since similar results were obtained with small-particle preparations from strain AN62. The percentage reduction of ubiquinone in small-particle preparations from this strain in the absence of substrate rose to about 50% on storage of the membranes at 0°C (A. M. Snoswell, unpublished work). The inhibition of reduction of ubiquinone by piericidin A has also been shown in ox heart mitochondria (Jeng *et al.* 1968) under conditions where, from the data presented, it is likely that reduction of the cytochromes occurred.

Scheme 1 is advanced as a basis for further work, taking into account the various observations reported above.

From the evidence discussed above, ubiquinone has been placed both before and after, cytochrome b_1 in the electron-transport chain. However, it is difficult to envisage ubiquinone functioning as a direct electron carrier at two sites, since its redox potential has not been demonstrated to be markedly changed by environment (see Boyer, 1968). The effects of the inhibitor piericidin A in fact suggest that ubiquinone is not acting as a direct electron carrier. Thus, although this inhibitor, with NADH as substrate, causes an increase in the percentage reduction of flavin and cytochrome b_1 in the steady state, it completely inhibits reduction of ubiquinone. However, lack of ubiquinone or the presence of piericidin A caused marked inhibition of forward electron transport. Therefore we propose that ubiquinone is complexed with an electron carrier and that the carrier alone does not function as efficiently in electron transport as the electron carrier–ubiquinone complex. Ubiquinone would be in the semiquinone form in the complex and would disproportionate on extraction into organic solvent giving about 50% of ubiquinol. The elimination of the electron-spin-resonance signal by piericidin A would reflect disruption of the complex with subsequent inhibition of electron transport. Estimation of the percentage reduction of ubiquinone in the membranes, after the addition of piericidin A, indicated that complete oxidation of the ubiquinol (formed in the membranes by disproportionation of ubisemiquinone after addition of inhibitor) took several minutes, even though the radical was eliminated in less than 30s. Thus piericidin A, as well as preventing reduction of ubiquinone on the addition of substrate, also prevents rapid oxidation of endogenous ubiquinol via the electron-transport chain, implying that piericidin A effectively separates ubiquinone from the remainder of the electron-transport chain.

The most likely electron carrier to form a complex



Scheme 1. Ubiquinone function in *E. coli*. Abbreviations: fp, flavoprotein; Q*, ubisemiquinone; cyt, cytochrome.

with ubiquinone would be non-haem iron, since non-haem iron can function at different redox potentials (see Lardy & Ferguson, 1969). An electron-spin-resonance signal attributed to non-haem iron has been found in membranes from strain AN 62 (Hamilton *et al.* 1970), and, further, iron analysis on membrane preparations (D. J. David, unpublished work) indicated that there was more than sufficient iron to allow formation of the proposed complex. A non-haem iron–ubiquinone–protein complex in mitochondria has been suggested (Vallin & Löw, 1968) as the primary high-energy intermediate in oxidative phosphorylation (see also Blumberg & Peisach, 1965; Moore & Folkers, 1964). In this regard it may be noted that an anaerobic-type growth yield is given by strain AN 59 (*Ubi*$^-$) even though low oxidase activities can be demonstrated. It is possible that evidence for an interaction between the ubisemiquinone and non-haem iron could be obtained from electron-spin relaxation studies similar to those carried out by Beinert and co-workers (see Beinert & Hemmerich, 1965) on flavin–metal systems.

The terminal region of the electron-transport chain has not been studied extensively in the present work because of the difficulty of measuring the steady-state oxidation–reduction levels of cytochrome *o* in the presence of cytochrome b_1. Cytochrome *o* is likely to be the important terminal oxidase in the membranes of *E. coli* cells harvested in the early stages of growth (Castor & Chance, 1959). Vitamin K has not been included in Scheme 1, although it has been suggested that this vitamin is the quinone involved in NADH oxidation in *E. coli* (Kashket & Brodie, 1963). Preliminary experiments with a vitamin K-deficient strain of *E. coli* indicate that this quinone is not concerned in aerobic respiration.

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