BBA 45609

THE USE OF A UBIQUINONE-DEFICIENT MUTANT IN THE STUDY OF MALATE OXIDATION IN *ESCHERICHIA COLI*

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(Received March 10th, 1967)
(Revised manuscript received June 12th, 1967)

SUMMARY

1. Malate oxidation catalyzed by sub-cellular fractions of a normal strain of *Escherichia coli* and a mutant strain unable to form ubiquinone has been compared.

2. The system catalyzing the aerobic oxidation of malate was localized in a membranous small particle fraction separated by $(NH_4)_2SO_4$ fractionation following disruption of the cells in a French pressure cell.

3. Comparison of malate oxidation catalyzed by particles from normal and mutant cells indicates that ubiquinone is concerned in malate oxidation. Malate oxidation proceeds at about half the normal rate in particles from cells lacking ubiquinone.

4. Malate oxidation catalyzed by small particles from cells lacking ubiquinone was insensitive to the low concentrations of dicoumarol which inhibited oxidation catalyzed by particles from normal cells. Malate oxidation catalyzed by particles from cells lacking vitamin K was even more sensitive to dicoumarol than that catalyzed by particles from normal cells. Therefore dicoumarol at low concentrations is not acting as a vitamin K antagonist.

INTRODUCTION

Ubiquinone is found in particulate structures such as mitochondria and bacterial chromatophores and this has led to the suggestion that the quinone is involved in electron transfer processes. The role played by ubiquinone in mitochondria has been subject to intensive research in recent years. (For summary and references see ref. 1.) The work of GREEN AND BRIERLEY[2] would suggest that ubiquinone plays a major part in electron transport processes while that of CHANCE and REDFEARN and their colleagues[3,4] suggests that ubiquinone may be concerned in reversed electron transport or a by-pass rather than on the main pathway of electron transport to oxygen. KRÖGER AND KLINGENBERG[5] suggest that ubiquinone is concerned in both forward and reversed pathways of electron transport.

Ubiquinone function in bacterial electron transport systems has not received

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as much attention as in the mitochondrial systems. KNOWLES AND REDFEARN[6] have examined ubiquinone function in *Azotobacter vinelandii* using a technique which has been used with mitochondria, namely acetone extraction. Although activity was lost following extraction, it was not possible to restore oxidative activity by adding ubiquinone. KASHKET AND BRODIE[7,8] have studied ubiquinone function in *Escherichia coli*, an organism which contains both ubiquinone and vitamin K and concluded that ubiquinone is involved in succinate oxidation while vitamin K is involved in NAD-linked substrate oxidations.

It has been suggested previously that multiple aromatic auxotrophs could be grown in such a way as to provide cells deficient in the quinones and that such cells might be of use in the study of quinone function[9]. A more satisfactory method would be to obtain mutants which could not form ubiquinone and compare such strains with normal cells. Such a mutant of *E. coli* K12 (AB3285), which is unable to carry out the first specific step in ubiquinone biosynthesis, namely the conversion of chorismate into 4-hydroxybenzoate, has been isolated[10]. Part of the procedure used for the isolation of the mutant strain unable to form ubiquinone was to select strains unable to use malate as sole carbon source. It was observed, however, that the mutant still oxidized malate, suggesting that energy yielding processes might have been affected. The following experiments examine the relationship between ubiquinone and malate oxidation using sub-cellular fractions of Strain AB3285 and a revertant (AB3290) obtained from it.

METHODS

Organisms and media

The strains of *E. coli* K12 used were AB3285, AB3290 and AB3291. These strains are described in detail elsewhere[10]. Briefly, Strain AB3285 is a mutant unable to use malate as sole carbon source and which does not form detectable amounts of ubiquinone. This strain forms about 5 times the amount of vitamin K found in normal cells. The mutation in Strain AB3285 affects the first specific step in ubiquinone biosynthesis, namely the conversion of chorismate to 4-hydroxybenzoate.

Strain AB3290 is a revertant obtained from AB3285 which simultaneously regained the ability to grow on malate medium and to form ubiquinone and will be referred to in this paper as the 'normal strain'.

Strain AB3291 is a mutant unable to form detectable amounts of vitamin K but forming about 3 times the normal level of ubiquinone.

Cells were grown in a 0.5 % (w/v) glucose–mineral salts medium[11].

Chemicals

Chemicals used were obtained commercially and were not further purified. Ubiquinone (Q-2) was kindly provided by Merck, Sharp and Dohme, U.S.A. and piericidin A by Prof. S. TAMURA, Department of Agricultural Chemistry, University of Tokyo. L-Malate was used throughout except where otherwise indicated.

Preparation of sub-cellular fractions

Cells were grown as 1-l cultures in 2-l flasks, shaken on a New Brunswick gyro rotary shaker at 37°. Cultures were harvested in mid-exponential phase (about

0.6 mg dry wt./ml) and washed once in about 200 ml of cold potassium phosphate buffer (0.1 M, pH 7.0) per l of culture. The washed cells were resuspended in the above buffer (1 ml buffer/0.5 g wet wt. cells) and smashed in a French pressure cell at 20000 lb/inch^2. Examination by phase contrast microscopy indicated that these conditions caused disruption of almost all of the cells. The disrupted cells were then centrifuged at 25000 $\times$ g for 15 min to remove any whole cells and large pieces of membrane. The deposit will be referred to as the 'large particle' fraction. A 'small particle' fraction was separated from the supernatant by adding solid $(NH_4)_2SO_4$ slowly to give 20 % satn. and the solution was stirred for a further 20 min to ensure equilibration. The precipitate was collected following centrifugation at 25000 $\times$ g for 15 min and resuspended in 1 ml of phosphate buffer (0.1 M, pH 7) for each original gram wet weight of cells. Solid $(NH_4)_2SO_4$ was then added to the supernatant to give 80 % satn. and the precipitate collected as above. All operations on the washed cells and cell fractions were carried out at 0–4°. Proteins were estimated with Folin's phenol reagent[12].

Determination of quinone content of particles

The large particle fraction and the small particle fraction (as the $(NH_4)_2SO_4$ precipitate) were placed in Soxhlet thimbles, and the quinones extracted and chromatographed as described previously[9]. The yellow quinone bands were scraped off and eluted with diethyl ether. Absorption spectra of the solutions were measured in 1-cm cells in a Cary Model 11 spectrophotometer between 230 and 360 mμ to confirm the identity of the quinones. The absorbance at 248 mμ was used to estimate vitamin K and that at 275 mμ to estimate ubiquinone.

Test for ubiquinone reduction

The degree of reduction of ubiquinone was estimated after determining total ubiquinone and oxidized ubiquinone according to the method of HOFFMANN *et al.*[13]. Incubations were carried out in volumes of 1.5 ml at 30° and the reaction stopped by the addition of 5 ml of petroleum ether (b.p. 40–60°)–methanol (60:40, v/v) as described by KRÖGER AND KLINGENBERG[5].

Methods for measuring oxygen uptake

Oxygen uptakes were measured either in the conventional Warburg apparatus or by use of an Oxygraph Model K recording oxygen electrode (Gilson Medical Electronics, Wisc., U.S.A.). The electrode assembly of the latter apparatus was modified as described by SNOSWELL[14] and a medium composed of 30 mM sodium–potassium phosphate (pH 7.4) and 7.5 mM $MgCl_2$ was used. Buffer solutions were calibrated for oxygen content according to the method of CHAPPELL[15].

Reduction of pyridine nucleotides and cytochromes in small particles

The small particle preparation (0.2 ml) was added to 0.8 ml potassium phosphate buffer (0.1 M, pH 7). Fluorescence (activation 350 mμ; fluorescence 440 mμ, uncorrected) was measured in an Aminco Bowman spectrofluorimeter to which was attached a Moseley *xy* recorder with a 50 sec/inch time base. After measuring fluorescence for about 40 sec, 20 μmoles of DL-malate in 0.1 ml water were added, the contents of the cuvette mixed and fluorescence measurement continued. Increase

in fluorescence was taken as indicating pyridine nucleotide reduction. The reduced pyridine nucleotide was readily oxidized by aerating the contents of the cuvette by shaking.

The concentrations of the cytochromes in a number of preparations of small particles were compared after suspension in phosphate buffer as above and addition of malate or sodium hydrosulfite. The Soret peak in difference spectra was measured in a Cary Model 11 spectrophotometer.

RESULTS

Malate oxidation in sub-cellular fractions

A simple procedure for isolating a sub-cellular fraction which contained the enzyme system catalysing the oxidation of malate to oxaloacetate with the concomitant reduction of oxygen was developed. The fractionation procedure employed is outlined in METHODS. The data in Table I shows that malate oxidation is localised primarily in the 0–20 % $(NH_4)_2SO_4$ fraction. This fraction (see below) was shown to contain membranous particles and will be referred to hereafter as the 'small particle fraction'. The 20–80 % $(NH_4)_2SO_4$ fraction, while possessing little malate oxidation activity itself, was capable of stimulating the oxidation rate of the small particles. No membranous structures were detected in electron micrographs of the 20–80 % $(NH_4)_2SO_4$ fraction.

TABLE I

MALATE OXIDATION BY SUB-CELLULAR FRACTIONS

Oxygen uptake rates were measured in a conventional Warburg apparatus in a total volume of 3 ml. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 15 mM $MgCl_2$ and 20 mM DL-malate. The small or large particles were added in a volume of 0.25 ml (2–3 mg protein). The reaction was followed for 30 min at 37°. (20–80) refers to the protein precipitated from cell extracts between 20 and 80 % satn. with $(NH_4)_2SO_4$ (see METHODS).

Expt. No.	*Organism used*	*Oxidation rate (mμgatoms O per min per mg protein)*				
		Large particles	*Small particles*	*(20–80)*	*Small particles + (20–80)*	*Large particles + (20–80)*
I	AB3290 (normal)	8	124	8	248	—
II	AB3290	20	116	0	272	52
III	AB3285 (mutant)	12	84	0	128	32
IV	AB3285	—	52	—	92	—

TABLE II

QUINONE CONTENT OF SMALL PARTICLES

Organism	*Quinone content (mμmoles/mg protein)*	
	Vitamin K_2	*Ubiquinone*
AB3285	20	Not detected (<0.2)
AB3290	4.5	2.2
AB3291	Not detected (<0.05)	6.0

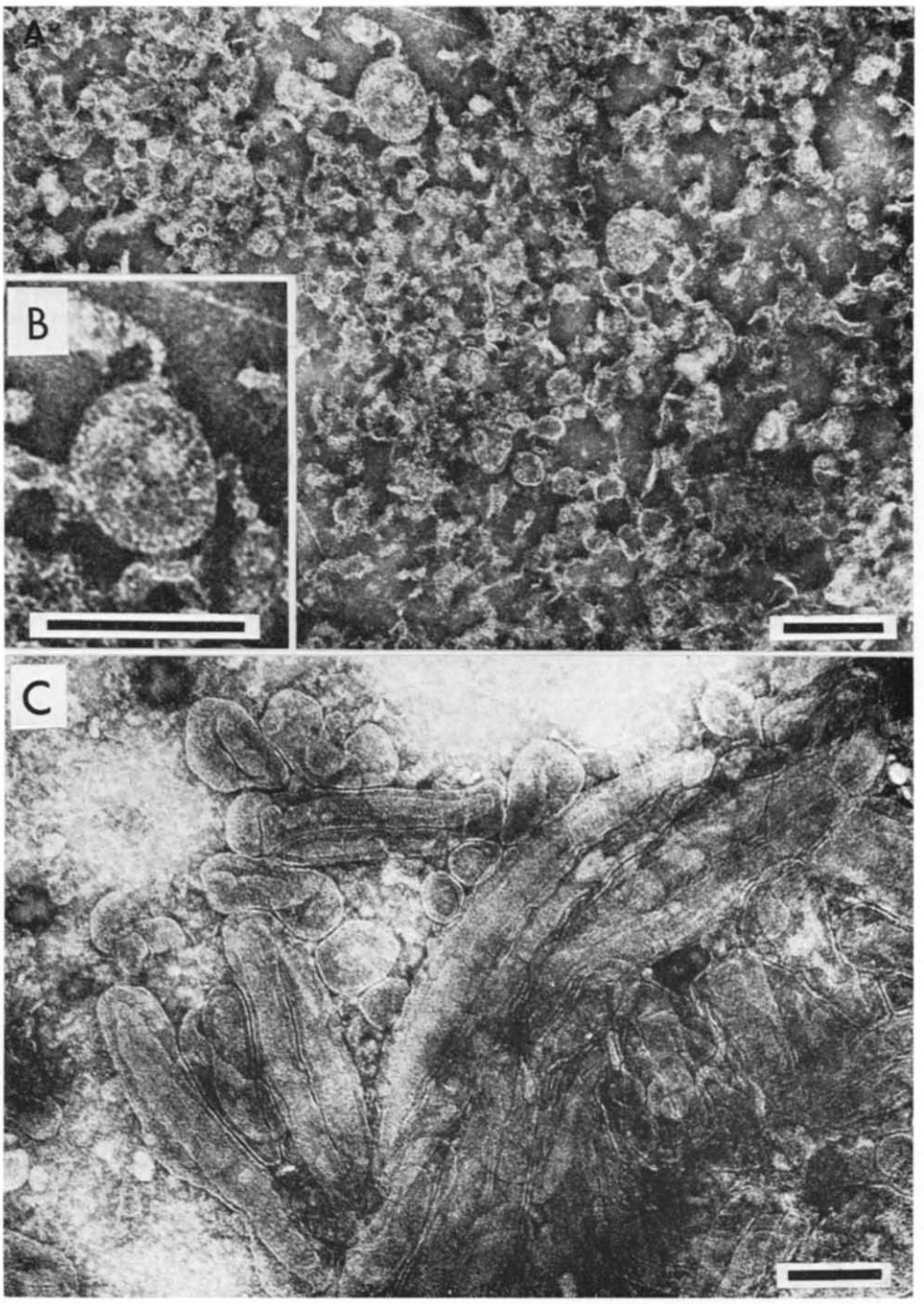

Fig. 1. Electron micrographs of small and large particle fractions. Fractions were negatively stained with 2 % potassium phosphotungstate (pH 7.0). A and B, small particle fraction; C, large particle fraction. Magnification marker in all cases = 0.2 μ.

Some properties of the small particle fraction

Electron microscopy

Examination of the small particle fraction from AB3290 by electron microscopy (Fig. 1) indicated the presence of membranous structures. The appearance of these structures was quite distinct from those found in the large particle fraction (Fig. 1) and resembled structures thought to be concerned in electron transport in mitochondria[16] and described in another bacterium[17].

Quinone content

An estimation of the quinone content of the particulate fractions from AB3290 indicated that the ratio of vitamin K to ubiquinone was constant at about 2 in both the small and large particle fractions. The small particles possessed 3 times the quinone content of the large particles. (Table II).

Substrates oxidized

The small particles from both the normal cells AB3290 and from the mutant AB3285 were capable of oxidizing a variety of substrates as shown in Table III. In general the oxidation rates of these substrates by the particles from the mutant were about a half of the rates with the normal particles with the exception of α-glycerophosphate. Pyruvate, succinate, β-hydroxybutyrate, oxaloacetate and glutamate were not oxidized to any significant extent by particles from either strain.

TABLE III

OXIDATION RATES OF VARIOUS SUBSTRATES WITH SMALL PARTICLES DERIVED FROM NORMAL *E. coli* AND A MUTANT UNABLE TO FORM UBIQUINONE

Rates of oxygen uptake were measured polarographically with an oxygen electrode in a volume of 2.0 ml at 30°. The reaction mixture contained 30 mM phosphate buffer (pH 7.4) and 7.5 mM $MgCl_2$ and small particles (0.7–1.4 mg protein). Substrates were added in a volume of 10 μl to give a final concentration of 2 mM.

Substrate added	*Number of experiments*	*Oxidation rate (mμgatoms O per min per mg protein)*	
		Normal strain	*Mutant strain*
None	6	Nil	Nil
α-Glycerophosphate	3	47	32
L-Malate	6	98	41
L-Lactate	3	131	76
Formate	2	158	81
$NADH_2$	1	435	180

Stoicheiometry of malate oxidation

As this study was concerned with the oxidation of malate by *E. coli* K12 it was necessary to establish that the oxygen uptakes observed when malate was used as the substrate were only due to the oxidation of malate and not subsequent reaction products. Table IV shows a stoicheiometric balance between oxaloacetate formed and oxygen utilized with normal particles of *E. coli* K12, indicating only a one-step oxidation was studied. Similar results were obtained for particles derived from the ubiquinone-deficient mutant.

TABLE IV

BALANCE BETWEEN OXALOACETATE FORMED AND OXYGEN UPTAKE DURING THE OXIDATION OF L-MALATE BY SMALL PARTICLES DERIVED FROM NORMAL *E. coli*

The reaction was started by the addition of 1.2 μmoles of malate and allowed to proceed for about 4 min at 30° in a volume of 2.4 ml. The reaction was then stopped by the addition of 0.2 ml of 40% trichloroacetic acid. Oxaloacetate was determined on the neutralized supernatant by the method of HORST AND REIM[18]. The reaction mixture as described in Table III.

Expt. No.	*Oxaloacetate formed (mμmoles/mg protein)*	*Oxygen used (mμgatoms/mg protein)*
I	242	237
II	215	212
III	223	219
IV	348	367

Malate-dependent reduction of bound pyridine nucleotides

A fluorimetric method (see METHODS) was used to test for substrate-dependent reduction of bound pyridine nucleotides in the small particles and in this way the small particles from the ubiquinone mutant and from the revertant could be compared. It was consistently found that higher levels of reduced pyridine nucleotides were detected in the particles from the mutant cells when they were incubated with malate than were detected in the particles from the normal cells. The addition of NAD to freshly prepared particles did not increase the amount of reduced pyridine nucleotide observed.

Role of ubiquinone in malate oxidation

The results shown in Table III indicate that, on the average, malate was oxidized by particles derived from the ubiquinone-deficient mutant at about half the rate observed with particles from normal cells. This is also illustrated in the experiment depicted in Fig. 2b. Addition of ubiquinone (Q-2) to particles from the mutant increased the rate of malate oxidation some 150% while the oxidation rate of normal particles was only increased 30% by a similar addition (Fig. 2b). In contrast, the addition of menadione, at five times the level of ubiquinone (Q-2) used, to particles from mutant cells increased the oxidation rate only 10%. These observations would indicate that in the particles from mutant cells lack of ubiquinone is the rate-limiting factor in the malate-dependent oxygen uptake. Estimation of bound pyridine nucleotide (see earlier) and of cytochrome levels (see METHODS) in the particles from normal and mutant cells support this conclusion. Thus pyridine nucleotides reducible in the presence of malate are at much higher levels in particles from mutant cells than in those from normal cells and levels of cytochromes are similar in particles from both types of cells.

Furthermore, examination of the ubiquinone (Q-8) present in the normal particles showed that it was all present in the oxidized form in the absence of added substrate, but after incubation of the particles with malate at 30° for 2 min some 35% of the total ubiquinone (Q-8) was reduced.

The effect of inhibitors on the oxidation of malate

The oxidation of malate by particles derived from the ubiquinone-deficient

mutant was not affected by dicoumarol at a concentration of 5 μM whereas the oxidation rate by particles from the normal cells was inhibited 56 % (Table V). This inhibition was only slightly reversed by ubiquinone (Q-2) (Table V).

The oxidation of malate by particles from the normal cells was also considerably more sensitive to inhibition by piericidin A than the oxidation by particles from the ubiquinone-deficient mutant. Subsequent addition of ubiquinone (Q-2) completely reversed the inhibition by piericidin A (Table V). The oxidation of malate by particles from both normal and ubiquinone-deficient cells was substantially inhibited by both 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and KCN and was not inhibited by antimycin A.

TABLE V

THE EFFECT OF VARIOUS INHIBITORS ON THE OXIDATION OF MALATE BY SMALL PARTICLES DERIVED FROM NORMAL *E. coli* AND A MUTANT UNABLE TO FORM UBIQUINONE

Rates of oxygen uptakes were measured as described in Table III. Piericidin A, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide or antimycin A were added in pure ethanol. The final ethanol concentration did not exceed 0.5 % and corrections for the effect of ethanol were made where required.

Inhibitor	*Final concn.*	% *Inhibition of oxidation rate*	
		Normal strain	*Mutant strain*
Dicoumarol	5 μM	56	Nil
Dicoumarol then Q-2 (0.11 mM)	5 μM	45	—
Piericidin A	10 μM	45	20
Piericidin A	20 μM	59	23
Piericidin A	40 μM	73	32
Piericidin A then Q-2 (0.11 mM)	20 μM	Nil	—
2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide	0.5 μg/ml	63	66
2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide	2.5 μg/ml	81	100
KCN	10 μM	100	75
Antimycin A	2.5 μg/ml	Nil	Nil

Further studies on the effects of dicoumarol on malate oxidation

As indicated in Table V 5 μM dicoumarol inhibited malate oxidation catalyzed by small particles from normal *E. coli* K12 cells by 56 %. The same concentration of dicoumarol did not inhibit the oxidation catalysed by small particles from a mutant strain unable to form ubiquinone (Fig. 2a). This mutant strain contains a derepressed level of vitamin K_2 (MK-8) (ref. 10).

The addition of ubiquinone (Q-2) to particles from the mutant deficient in ubiquinone restored the rate of malate oxidation to that catalyzed by particles from normal cells. Under these conditions 5 μM dicoumarol inhibited malate oxidation catalyzed by particles isolated from both the normal strain and ubiquinone-deficient mutant to the same extent (Fig. 2b). In contrast, added menadione had little effect on the oxidation rate catalyzed by particles from the mutant strain or on the dicoumarol inhibition.

The comparative insensitivity of particles from the ubiquinone-deficient mutant to inhibition by dicoumarol, in the absence of added ubiquinone, was seen over a wide range of concentrations of the inhibitor (Fig. 3). Furthermore malate

oxidation catalysed by particles from the mutant Strain AB3291 containing no vitamin K_2 (MK-8), but containing a derepressed level of ubiquinone (Q-8), was even more sensitive to inhibition by dicoumarol than the oxidation catalysed by particles from normal cells (Fig. 3).

Further evidence concerning the action of dicoumarol is provided by the experiments illustrated in Fig. 4. In these experiments the addition of ubiquinone (Q-2) to particles from the ubiquinone-deficient mutant not only restored oxidative

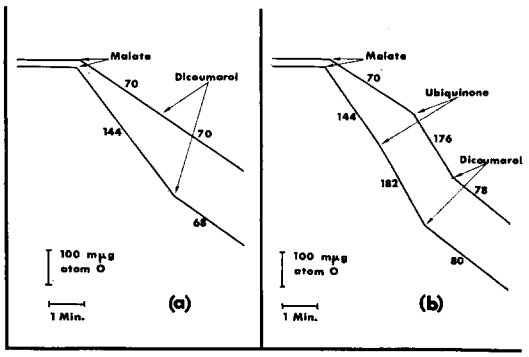

Fig. 2. The rate of malate oxidation catalysed by small particles derived from a normal and a ubiquinone-deficient strain of *E. coli* K12 and the effect of dicoumarol. The rate of oxygen uptake was measured with a recording oxygen electrode in a final volume of 2.0 ml at 30°. The reaction mixture contained 30 mM sodium phosphate buffer (pH 7.4), 7.5 mM $MgCl_2$ and small particles (0.9 mg protein); 4 μmoles DL-malate, 0.22 μmole Q-2 and 10 mμmoles of dicoumarol were added in 10-μl volumes where indicated. The upper line represents the rate of oxidation with particles from the ubiquinone-deficient mutant and the lower line the normal strain. The numbers represent rates of oxygen uptake in mμgatoms/min per mg protein.

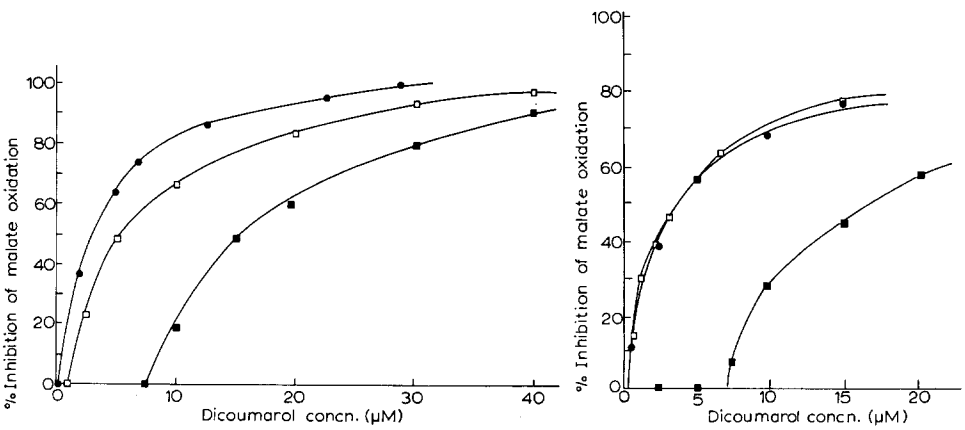

Fig. 3. Dicoumarol inhibition of malate oxidation catalysed by small particles from three strains of *E. coli*. Measurements and experimental conditions as in Fig. 2. ■—■, figures obtained with the mutant strain containing no ubiquinone; □—□, obtained for the normal strain; ●—●, obtained for the mutant containing no vitamin K_2.

Fig. 4. Dicoumarol titration curves for the inhibition of malate oxidation catalysed by small particles from a normal strain and ubiquinone-deficient mutant of *E. coli*. Measurements and experimental conditions as in Fig. 2. ■—■, figures obtained for the ubiquinone-deficient strain; □—□, obtained for the normal strain; ●—●, obtained for the ubiquinone-deficient strain where the malate oxidation rate had been restored by the prior addition of 0.33 μmole Q-2.

activity (see Fig. 2b) but also restored the sensitivity to dicoumarol to normal levels over a wide range of concentrations of inhibitor.

DISCUSSION

Studies on the role of ubiquinone in metabolism have, in general, been carried out either by experiments on the kinetics of oxidation–reduction changes in components of the respiratory chain or by the destruction or removal of ubiquinone from mitochondria or bacterial respiratory particles and restoration of activity by added ubiquinone. The latter techniques suffer from certain inherent difficulties. Extraction of mitochondria or other membranous structures with lipid solvents must necessarily lead to damage to the membranes. Irradiation to destroy quinones also leads to difficulties of interpretation, particularly in the case of the mixed quinone system in *E. coli* in which both ubiquinone and vitamin K are present.

In the present experiments a new approach to the study of ubiquinone function is presented. This approach involves a comparison of metabolic reactions which might involve ubiquinone in cells of a normal organism compared with a mutant of the same organism which has lost the ability to make ubiquinone. Such comparisons in the present paper are carried out between a mutant which lacks the ability to carry out the first specific reaction in ubiquinone biosynthesis and a revertant, which is taken as normal *E. coli*, obtained from that mutant.

It is clear that the system catalysing the aerobic oxidation of malate in *E. coli* K12 grown under the conditions described is localised in the small particle fraction. KASHKET AND BRODIE[8] working with *E. coli* W, found an approximately equal distribution of malate oxidation activity between the large and the small particles. This discrepancy may be due to the fact that in the present study glucose was used as carbon source for growth of the cells whereas KASHKET AND BRODIE[8] used either succinate or malate.

The nature of the factor in the 20–80 % $(NH_4)_2SO_4$ fraction stimulating malate oxidation has not been studied in detail. However, preliminary experiments show that freshly prepared small particles alone are incapable of reducing added (as distinct from endogenous) NAD in the presence of malate. However, if the 20–80 % $(NH_4)_2SO_4$ fraction is added the resultant system is then capable of reducing added NAD suggesting that there is a soluble malic dehydrogenase in the proteins precipitated at the higher concentrations of $(NH_4)_2SO_4$. A soluble malic dehydrogenase precipitated by high $(NH_4)_2SO_4$ concentrations has been reported in *A. vinelandii*[19].

The results of JONES AND REDFEARN[19] with particles from *A. vinelandii* suggested that pyridine nucleotides were not involved in malate oxidation as added NAD failed to stimulate the oxidation rate and a quantitative estimate of bound NAD indicated that little NAD was present in their particles. Although a quantitative estimate was not made in the present study, the fluorimetric technique used suggested that added NAD could not reach the site of malate dehydrogenation. However, reduction of endogenous pyridine nucleotide by malate could be detected readily. Aged particles, while having a lower level of bound pyridine nucleotides reducible by malate were able to reduce added NAD.

The distribution of ubiquinone and vitamin K between the large and small particle fractions found in the present experiments is at variance with that found by KASHKET AND BRODIE[8]. In the present study the K level was higher than the ubi-

quinone in both particulate fractions, but the quinone content of the small particles reported by KASHKET AND BRODIE[8] indicated that K was approximately one-twentieth the concentration of ubiquinone. This discrepancy also is probably due to the different carbon sources used. Thus the work of POLGLASE, PUN AND WITHAAR[20] indicates that under aerobic conditions the ubiquinone level is high and the vitamin K level is low. Under anaerobic conditions the relative levels are reversed. Oxygen would be an important electron acceptor for the facultative anaerobe *E. coli* growing with succinate or malate as sole carbon source. Glucose on the other hand would allow growth under semi-aerobic conditions tending to equalize the concentrations of the quinones.

A considerable amount of experimental evidence is presented in this paper which clearly indicates ubiquinone is involved in the oxidation of malate in *E. coli* K12.

(a) The endogenous ubiquinone (Q-8) present in the small particles is reduced when these particles are incubated with malate. This is in contrast to observations of KASHKET AND BRODIE[7]. However, in the experimental system used by these workers there was a considerable degree of reduction even in the absence of malate owing to the presence of KCN in the incubation mixture. In the present work the ubiquinone (Q-8) was all in the oxidized form prior to the addition of malate and the maximum degree of reduction in the presence of malate was 35 %, equivalent to the endogenous reduction reported by KASHKET AND BRODIE[7].

(b) The rate of malate oxidation in the ubiquinone-deficient mutant was about 40 % of the rate in normal cells and this rate could be restored to the oxidation rate observed with normal cells by the addition of ubiquinone (Q-2). Further, the addition of ubiquinone (Q-2) at the same concentration to particles from normal cells had little effect indicating that artificial pathways of electron transport are probably not operating. This is supported by the observation that the addition of a similar concentration of menadione had little effect on the rate of malate-dependent oxygen uptake with particles derived from either mutant or normal cells.

(c) Piericidin A, which appears to be a specific inhibitor of ubiquinone[21,22], inhibits the oxidation of malate in the normal cells and this inhibition can be completely reversed by the addition of ubiquinone (Q-2).

(d) The oxidation of malate by small particles from the ubiquinone-deficient mutant is not inhibited by 5 μM dicoumarol, whereas the oxidation rate in particles from normal cells is inhibited 56 % at the same concentration. However, if the oxidation rate of the small particles from the mutant is raised to that of the normal cells by the prior addition of ubiquinone (Q-2) then the oxidation in both particles is equally sensitive to dicoumarol.

Thus the evidence cited above clearly indicates that ubiquinone is involved in one pathway of malate oxidation. The results presented in this paper also indicate that some oxidation of malate in this organism proceeds without the participation of ubiquinone.

It is significant that of the three strains of *E. coli* K12 examined, the malate-oxidizing system of the mutant containing ubiquinone (Q-8), but no vitamin K_2 (MK-8), was the most sensitive to dicoumarol inhibition. In contrast, the malate-oxidizing system in the mutant containing no ubiquinone (Q-8), but containing vitamin K_2 (MK-8), was the least sensitive of the three strains examined. This indicates that, at low concentrations, dicoumarol preferentially inhibits the ubiquin-

one-dependent pathway at a site not involving vitamin K. It might be assumed that as the malate-oxidizing system in the mutant containing no ubiquinone, but containing vitamin K, is inhibited by dicoumarol at higher concentrations, vitamin K may be involved in malate-dependent oxygen uptake. While this may be true, no experimental evidence is provided in the present work to support this conclusion.

Dicoumarol has long been regarded as a vitamin K antagonist. In the present experiments, while the exact site of inhibition of oxygen uptake by low concentrations of dicoumarol has not been determined, it is not acting as a vitamin K antagonist. Another compound previously regarded as a typical vitamin K antagonist, namely SN5949, inhibits the succinoxidase system in animal mitochondria[23]. This system requires ubiquinone rather than vitamin K for activity. The present results would reinforce the suggestion[24] that a number of previous conclusions regarding functions attributed to vitamin K based on inhibitor studies may need to be revised. The availability of mutant strains of *E. coli*, lacking ubiquinone or vitamin K, provides useful experimental systems for the further investigation of the function of both ubiquinone and vitamin K.

ACKNOWLEDGEMENTS

We are grateful to Dr. I. HOLMES for the electron microscopy and valuable discussion and to Miss L. PURVIS for technical assistance. The Australian National Health and Medical Research Council, the Australian National Heart Foundation and the Institute af Arthritis and Metabolic Diseases of the National Institutes of Health, Washington, U.S.A. (Grant AM04632) are thanked for research grants.

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