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STUDIES ON THE ELECTRON TRANSPORT SYSTEM

XXIII. COENZYME Q OXIDASE

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SUMMARY

The preparation and properties of a submitochondrial enzyme system containing predominantly cytochrome *a* have been described. This system is capable of catalyzing the oxidation of reduced coenzyme Q by molecular oxygen. This particulate enzyme requires the presence of both cytochrome *c* and a lipoprotein of mitochondrial origin in order to catalyze the above reaction. The results presented in this communication suggest that coenzyme Q is situated in the aforementioned lipoprotein between cytochromes *b* and *c*.

The abbreviations used in the text are as follows: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; ADP, adenosine diphosphate; Tris, Tris/hydroxymethyl)aminomethane; and QH_2, the reduced form of coenzyme Q. Coenzyme Q_{10}[12] was the homologue used throughout this investigation.

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INTRODUCTION

Previous communications from this laboratory have shown that coenzyme Q, a newly recognized lipid quinone of mitochondrial origin, is a member of the terminal electron-transport system[1-5] which undergoes in the mitochondrion rapid reduction in presence of substrates such as the citric acid cycle intermediates, β-hydroxybutyrate, and DPNH. Its reduced form (QH_2) is oxidized rapidly when mitochondria are incubated under aerobic conditions. These reactions of bound coenzyme Q were also studied in several submitochondrial particles. In addition, similar oxido-reduction reactions were shown to occur when purified coenzyme Q was added to a system containing catalytic quantities of mitochondrial particles[2,3]. It was also demonstrated that mitochondrion-bound coenzyme Q undergoes oxido-reduction during the process of phosphorylative electron transport[1]. The oxido-reduction behavior of coenzyme Q is influenced by the presence or absence of inorganic phosphate and ADP. Thus, in absence of added ADP, inorganic phosphate greatly diminishes the rate of oxidation of mitochondrion-bound QH_2. This condition of arrested electron transport due to absence of a high-energy phosphate acceptor can be corrected by ADP, in presence of which QH_2 is rapidly oxidized[3]. These results, therefore, indicated that this newly discovered compound is a member of the electron-transport system of mitochondria which may play a significant role in phosphorylation.

In the present investigation, an attempt was made to isolate from the mitochondrial electron-transport chain the simplest segment which was capable of catalyzing the oxidation of QH_2 by molecular oxygen. Previous work[3] had shown that the reduction of mitochondrion-bound coenzyme Q was inhibited by antimycin A. This finding indicated that coenzyme Q was situated on the oxygen side of cytochrome *b*, since according to GREEN *et al.*[6] as well as CHANCE and his collaborators[7,8] the reduction of cytochrome *b* is not inhibited by antimycin A. Therefore, a knowledge of the components required for the oxidation of QH_2 would further assist in localizing this compound relative to other members of the electron-transport system. The present communication describes the preparation and properties of a particle derived from heart mitochondria which catalyzes the oxidation of QH_2 by molecular oxygen. This particle contains essentially cytochrome *a* and very little red hemes. It requires the presence of cytochrome *c* as well as a lipoprotein, isolated from mitochondria by BASFORD AND GREEN[9], in order to catalyze the oxidation of QH_2 by molecular oxygen.

METHODS

Preparation of the enzyme system

The enzyme system can be prepared by two different methods which will be referred to as preparations I and II.

Preparation I. The electron-transport particle (ETP) is isolated from crude homogenates of beef-heart mitochondria in the same manner as described previously[10]. This particle is then treated with deoxycholate (0.5 mg/mg protein) in presence of Tris buffer, pH 8.0, and the green and red fractions of ETP are separated by centrifugation. The properties of these two fractions have already been described[11]. The 0.25 *M* sucrose suspension of the green fraction of ETP is homogenized, adjusted to a protein concentration of 10 mg/ml, and mixed with 0.1 vol. 2.5 *M* sodium acetate

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buffer, pH 5.7. The mixture is immediately centrifuged for 5 min at 30,000 rev./min in the No. 40 rotor of the Spinco preparative centrifuge. After centrifugation, the supernatant fluid is discarded and the residue is suspended in an amount of 0.125 *M* sucrose (0.1 *M* with respect to Tris chloride, pH 8.0) equal to the volume of the discarded supernatant. The suspension is thoroughly homogenized. Then, deoxycholate (10 %, pH 7.5) is added with stirring until a concentration of 1 mg deoxycholate/mg protein is reached. The partially clarified suspension is centrifuged for 5 min at 10,000 rev./min in the No. 40 rotor of the Spinco centrifuge. The clear, supernatant fluid is then decanted carefully and recentrifuged in the same rotor for 45 min at 40,000 rev./min. The sediment which forms after centrifugation is collected and suspended in 0.25 *M* sucrose. All the above operations should be carried out at 0°. The yield of the product, as calculated from the total protein content, is about 12 to 13 % by weight of the protein of the starting suspension of ETP.

Preparation II. A suspension of beef-heart mitochondria in 0.25 *M* sucrose is adjusted to a protein concentration of 15 mg/ml and acidified to pH 5.7 by addition of 2.5 *M* sodium acetate buffer, pH 5.7. The mixture is immediately centrifuged for 5 min at 15,000 rev./min in the No. 30 rotor of the Spinco centrifuge. The supernatant fluid is discarded and the sediment taken up in a mixture of equal volumes of 0.2 *M* Tris, pH 8.0 and 0.25 *M* sucrose. The suspension is thoroughly homogenized and brought to the original volume with the Tris–sucrose mixture. Then, deoxycholate is added to a concentration of 1 mg/mg protein, and the partially clarified suspension is centrifuged for 5 min at 15,000 rev./min in the No. 30 rotor of the Spinco centrifuge. The clear, supernatant fluid is further centrifuged for 60 min at 30,000 rev./min, whereupon the red hemoproteins remain in solution while the coenzyme Q oxidase system sediments. The green sediment is taken up in 0.25 *M* sucrose. The suspension is homogenized and adjusted to a protein concentration of 10 mg/ml. The treatments with acetate buffer and deoxycholate are once again repeated on this fraction. The yield of the second green sediment on the basis of total protein is about 10 % of the original mitochondrial suspension.

Both preparations I and II are yellowish-green in color, form an opalescent suspension in 0.25 *M* sucrose, and can be stored conveniently at −20° with little loss of activity. In order to distinguish the above preparations from other mitochondrial derivative particles mentioned in this communication, they shall be referred to henceforth as coenzyme Q oxidases I and II.

The assay system

The assay of enzymic activity was carried out as follows. In a glass-stoppered test tube were placed 50 μmoles Tris-chloride, pH 7.5, 0.02 mg cytochrome *c*, 0.4 mg* lipoprotein[8], about 150 to 200 mμmoles QH_2 dissolved in 0.02 ml absolute ethanol, and 0.25 *M* sucrose to a final volume of 1.0 ml. The enzyme was added last and the tubes were incubated at 38° for the periods of time indicated in the respective tables. The gas phase was air. After incubation, the tubes were withdrawn from the bath and immediately 1 ml 0.1 *N* $HClO_4$ was added to each tube in order to terminate the reactions by denaturing the enzymes. Then, 1 ml 95 % ethanol and 2 ml of "spectro-grade" cyclohexane were added to each tube. They were then stoppered

* The weight refers to the total dry weight of the lipoprotein including lipid.

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and placed on a horizontal, reciprocal shaking-machine for 45 min. Addition of ethanol helps the extraction of coenzyme Q into cyclohexane. The contents of the tubes (after shaking) were transferred to conical test tubes and these were centrifuged in a clinical centrifuge for 2 min. Then, the clear cyclohexane layer was withdrawn and the spectrum of coenzyme Q in cyclohexane was recorded by the DK-2 Beckman spectrophotometer. Calculation of the degree of oxidation of QH_2 from such spectra has been described in a previous communication[3].

Preparation of reduced coenzyme Q

Crystalline coenzyme Q (3 to 5 mg) was placed in a glass-stoppered test tube and dissolved in 1 ml absolute ethanol. Then, 3 mg solid KBH_4 were added and the tube was flushed with nitrogen. Upon reduction by KBH_4, the golden yellow color of coenzyme Q diminished and the solution becomes a very pale yellow. At this point 0.1 ml 6 *N* HCl was added to the tube in order to decompose excess KBH_4. The presence of acid also stabilizes the QH_2. Then, 1 ml water and 2 ml cyclohexane were added and the mixture was shaken for a few minutes in order to extract the QH_2 into cyclohexane. The cyclohexane layer was then withdrawn, evaporated under vacuum to dryness and the reduced quinone was taken up in absolute ethanol to the desired concentration. QH_2 in ethanol is slowly oxidized in air. Therefore, the preparations were either kept under nitrogen or used immediately. The molar concentration of coenzyme Q was calculated on the basis of $E^{1\%}_{1cm} = 165$ for coenzyme Q at 275 mμ and the approximate molecular weight of 850.

Estimation of the coenzyme Q content of the particulate enzymes

In a previous communication, a method was described for the extraction of coenzyme Q in which the particles were heated at about 90° in presence of 0.5 *M* KH_2PO_4 prior to extraction with cyclohexane[5]. This procedure, although satisfactory for mitochondria and some of the derivative particles, fails completely when applied to coenzyme Q oxidase. Consequently the procedure described below was used in this case. About 30 mg of particle protein suspended in 3 ml 0.25 *M* sucrose were placed in a glass-stoppered test tube. Then, 150 mg pyrogallol and 5 ml of freshly prepared 10 % KOH in ethanol (95 %) were added. The tube was fitted with a reflux condenser (a conical centrifuge tube packed with ice and placed on top of the test tube so that the tapered end hangs inside, works equally well) and the mixture was refluxed at 85° for 30 min. The tube was then cooled to room temperature and the contents extracted three times with 3 ml each of "spectro-grade" cyclohexane. The cyclohexane extracts were combined and washed twice by shaking in a separatory funnel with 5 ml each of water. Then, the cyclohexane solution was transferred to a round-bottom flask and the solvent was removed under vacuum. The material which remained in the flask was taken up in a known volume of absolute ethanol and its spectrum recorded before and after addition of KBH_4. The spectrum of such extracts shows the characteristic ultraviolet spectrum of coenzyme Q with a single peak of absorption at 275 mμ. Upon addition of a few grains of solid KBH_4, the characteristic absorption spectrum of QH_2 with a peak at 290 mμ appears. The concentration of coenzyme Q was calculated from the difference in the absorptions of oxidized and reduced coenzyme Q at 275 mμ. The $E^{1\%}_{1cm}$ value for the difference in the absorptions of oxidized and reduced coenzyme Q at 275 mμ has been found[12] to be 142.

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MATERIALS

Cytochrome *c* was obtained from Sigma Chemical Co., deoxycholic acid from Matheson Coleman and Bell Division of Matheson Company, Inc. and "spectrograde" cyclohexane from Eastman-Kodak Co. Coenzyme Q was prepared according to the procedure already described[13] and was supplied by the Merck Sharpe and Dohme Laboratories. The lipoprotein was kindly provided by Dr. R. E. BASFORD and cytochrome c_1 by Dr. D. E. GREEN.

RESULTS

Activity and requirements of the enzyme system

As was pointed out earlier, preparations I and II of the enzyme require the presence both of cytochrome *c* and of the mitochondrial lipoprotein[9] in order to catalyze the oxidation of QH_2 by molecular oxygen. Table I shows that mitochondria are capable of catalyzing the oxidation of QH_2 without any additions, the presence of cytochrome *c* and lipoprotein improving slightly the activity of the system. By comparison, coenzyme Q oxidase II is completely devoid of activity unless both cytochrome *c* and the lipoprotein are added. In the supplemented system, fresh preparations of coenzyme Q oxidase II are capable of catalyzing the oxidation of about 340 mμmoles QH_2/min/mg enzyme protein at 38°.

TABLE I

THE REQUIREMENTS OF COENZYME Q OXIDASE II

Expt.	*System*	*mμMoles of QH_2 oxidized*	
		*mitochondria**	*coenzyme Q oxidase*
1	Enzyme	27	0
	Enzyme + lipoprotein	41	0
	Enzyme + cytochrome *c*	34	5.7
	Enzyme + lipoprotein + cytochrome *c*	45.5	44.7
2	Enzyme	30.5	2.5
	Enzyme + lipoprotein + cytochrome *c*	54.6	131

Assay conditions: 50 μmoles Tris chloride, pH 7.5; 105 mμmoles QH_2; 0.1 mg enzyme protein; and where indicated, 0.4 mg lipoprotein and 0.02 mg cytochrome *c*. Duration of incubation at 38° was 1.5 min in Expt. 1 and 4 min in Expt. 2.

* Heavy beef-heart mitochondria[22] were used in this experiment.

Coenzyme Q oxidase I, which is obtained from ETP, is much more active than preparation II. The former can catalyze the oxidation of about 320 mμmoles QH_2/min/mg protein at 38° (Fig. 3). Figs. 1 and 2 show the activity of this system as a function of the concentration of cytochrome *c* and the lipoprotein. The enzyme preparation is incapable of catalyzing the oxidation of QH_2 in the absence of either of these two components. However, in presence of catalytic amounts of cytochrome *c* and the lipoprotein, a substantial degree of oxidation occurs. As seen in Fig. 1, there is a rapid rise in the rate of oxidation of QH_2 as the amount of added cytochrome *c* is increased to about 0.02 mg in the test system, but beyond this level increase of cytochrome *c* has only a slight effect. In the case of the lipoprotein also (Fig. 2), a

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rapid rise in activity is observed as the level of the lipoprotein is increased to about 0.2 to 0.4 mg in the assay system, while beyond this range of concentration addition of more lipoprotein becomes increasingly inhibitory. Fig. 3 shows the linear relationship between the concentration of enzyme and the rate of oxidation of the reduced quinone. Cytochrome *c* cannot be replaced by a purified preparation of lipid-free cytochrome c_1[11]. Nor does the addition of cytochrome c_1 along with cytochrome *c* stimulate the rate of oxidation of QH_2. The absolute lack of a stimulatory effect by cytochrome c_1 suggests that in this system cytochrome c_1 is not involved in the transfer of electrons from coenzyme Q to oxygen (Table II, Expt. 2). The lipid-soluble form of cytochrome *c*, recently described by WIDMER AND CRANE[15], is capable of replacing in part both the lipoprotein and cytochrome *c*. As seen in Expt. 1 of Table II, the absolute requirement of the system for both cytochrome *c* and the lipoprotein could be satisfied to some extent by the addition of lipid cytochrome *c* alone.

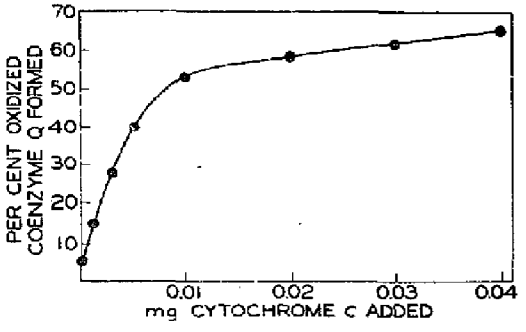

Fig. 1. Effect of concentration of cytochrome *c*. Conditions: 150 mμmoles QH_2; 0.1 mg coenzyme Q oxidase 1 protein; 50 μmoles Tris chloride, pH 7.5; 0.4 mg lipoprotein; and cytochrome *c* as indicated. The tubes were incubated for 57 sec at 38°.

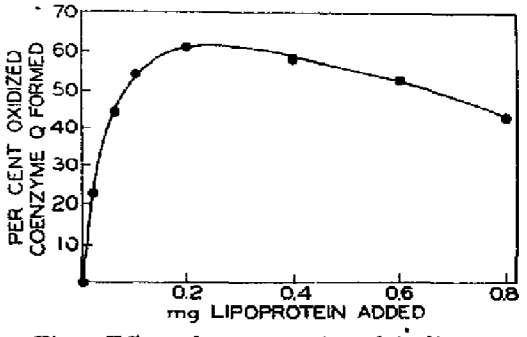

Fig. 2. Effect of concentration of the lipoprotein. Conditions: 150 mμmoles QH_2; 0.1 mg coenzyme Q oxidase 1 protein; 50 μmoles Tris chloride, pH 7.5; 0.02 mg cytochrome *c*; and lipoprotein as indicated. The tubes were incubated for 75 sec at 38°.

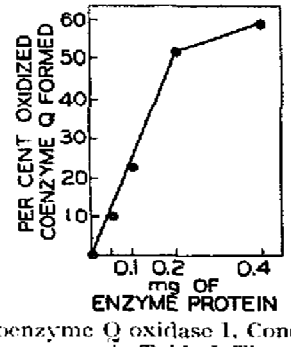

Fig. 3. Concentration curve for coenzyme Q oxidase 1. Conditions: 178 mμmoles QH_2. Additions of buffer, lipoprotein, and cytochrome *c* as in Table I. The tubes were incubated for 30 sec at 38°.

It may be added that lipid cytochrome *c* can be made by mixing water-soluble cytochrome *c* with the lipoprotein and extracting the lipid cytochrome *c* formed by heptane[9]. The above results suggest that the water-soluble form of cytochrome *c* perhaps cannot react with coenzyme Q, which is a lipid material utterly insoluble in water (see also Table V). This interaction occurs probably in the milieu of the

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TABLE II

EFFECTS OF LIPID CYTOCHROME *c* AND CYTOCHROME c_1

Expt.	*Additions*	*% QH_2 oxidized*
1	None	0
	Cytochrome *c*	0.5
	Lipoprotein	3
	Cytochrome *c* + lipoprotein	67.5
	Lipid cytochrome *c*	31
	Lipid cytochrome *c* + lipoprotein	33
2	None	0
	Cytochrome *c* + lipoprotein	43.5
	Cytochrome c_1 + lipoprotein	3
	Cytochrome *c* + cytochrome c_1 + lipoprotein	24

Assay conditions: 140 mμmoles QH_2, 0.4 mg coenzyme Q oxidase I protein and lipid cytochrome *c* and cytochrome c_1 in amounts equivalent to cytochrome *c*. Other additions as in Table I. Duration of incubation at 38° was 30 sec in Expt. 1 and 45 sec in Expt. 2.

lipoprotein, where cytochrome *c* assumes a lipid character. It should be pointed out that the lipoprotein contains a very high amount of coenzyme Q (about 7.5 mg/g dry weight) and can be isolated from mitochondria with bound cytochrome *c**.

ETP, the particle from which preparation I is derived, shows no requirement for added cytochrome *c* in catalyzing the oxidation of QH_2. However, as in the case of mitochondria, a small and variable degree of stimulation in the rate of oxidation of QH_2 by ETP is observed when the system is supplemented with the lipoprotein (Table III). After ETP is treated with deoxycholate, the green fraction which is obtained shows an absolute requirement for cytochrome *c* in the oxidation of QH_2 but still has only a partial requirement for the lipoprotein (Table III). Thus, it seems that the pH 5.7 treatment is a necessary preliminary for achieving an absolute requirement for the lipoprotein. Detailed studies on the oxido-reduction reactions of added coenzyme Q as catalyzed by mitochondria or ETP have been carried out by LESTER AND RAMASARMA and will be presented separately.

The coenzyme Q oxidase system is inhibited by antimycin A, KCN, NaN_3, or $ZnCl_2$. Fig. 4-a shows the inhibition of the oxidation of QH_2 by added antimycin A.

TABLE III

EFFECTS OF LIPOPROTEIN AND CYTOCHROME *c* WITH ETP AND THE GREEN FRACTION OF ETP

Enzyme	*% QH_2 oxidized*		
	Less lipoprotein	*Less cytochrome c*	*Complete system*
ETP	32	60.5	60.5
Green fraction	30.5	0	74

Assay conditions: 180 mμmoles QH_2 and 0.1 mg enzyme protein. Buffer, cytochrome *c* and lipoprotein additions as in Table I. The tubes were incubated for 1.5 min at 38°.

* The analytical studies of BASFORD[16] have shown that this lipoprotein contains 95% lipid and only 5% protein. Phospholipids compose about 95% of its lipid content. Purified lipoprotein migrates as a single peak when subjected to electrophoresis or ultracentrifugation[9].

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It is seen that very minute quantities of antimycin A are sufficient for nearly complete inhibition of the reactions. $ZnCl_2$ also is a potent inhibitor in the system. Fig. 4-b shows the effect of increasing concentrations of $ZnCl_2$ and the corresponding diminutions in the degree of oxidation of QH_2. As would be expected, cyanide completely inhibits the aerobic oxidation of QH_2, presumably by interacting with the cytochrome oxidase system of the enzyme preparation (Table IV). Azide also has some inhibitory effect when added at rather high concentrations. As seen in Table IV, Cu^{++} ions have a stimulatory effect in the system. Whether this stimulation is due to the effect of Cu^{++} on the reduction of cytochrome *c* by QH_2 or on the cytochrome oxidase system has not been investigated. It was shown, however, that copper does not affect a nonenzymic oxidation of QH_2.

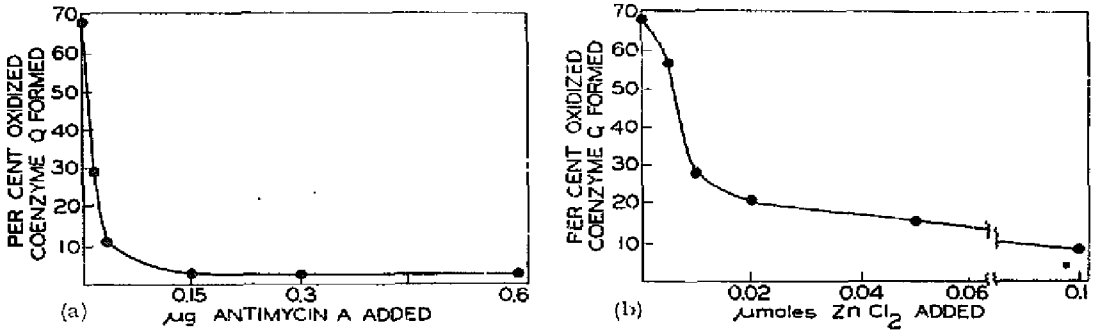

Fig. 4. Inhibition of coenzyme Q oxidase by (a) antimycin A and (b) $ZnCl_2$. Conditions: 147 mμmoles QH_2 and 0.1 mg coenzyme Q oxidase I protein. Additions of buffer, lipoprotein, and cytochrome *c* as in Table I. The tubes were incubated for 2.5 min at 38°.

TABLE IV

EFFECTS OF CYANIDE, AZIDE, AND COPPER

Expt.	*Additions*	*% QH_2 oxidized*
1	None	65
	KCN, 10 μmoles	2.5
2	None	63
	NaN_3, 20 μmoles	35
3	None	46
	$CuSO_4$, 0.1 μmole	51
	$CuSO_4$, 0.3 μmole	70
	$CuSO_4$, 0.5 μmole	88

Assay conditions: 150 mμmoles QH_2 and 0.1 mg coenzyme Q oxidase I protein. Buffer, cytochrome *c*, and lipoprotein additions as in Table I. Duration of incubation at 38° was 2.5 min in Expts. 1 and 2 and 1 min in Expt. 3.

As was pointed out in a previous communication[3], submitochondrial particles lacking the cytochrome oxidase system are capable of catalyzing the oxidation of externally added QH_2 by external ferricytochrome *c*. Concomitant with the oxidation of QH_2, cytochrome *c* becomes reduced. Therefore, it was of interest to determine whether the aforementioned lipoprotein catalyzes the interaction between QH_2 and

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cytochrome *c* or whether still another component, *e.g.* an enzyme, is required. This possibility was tested by adding QH_2 to a cuvette containing oxidized cytochrome *c* and measuring the rate of reduction of cytochrome *c* at 550 mμ. Table V shows that there is an insignificant rate of reduction of cytochrome *c* in such a system, which is definitely enhanced in presence of the lipoprotein. However, as it is seen in the last column of Table V, these rates are not at all comparable to the overall rate of oxidation of QH_2 as catalyzed by coenzyme Q oxidase. The rates in the latter system are several hundred-fold faster than the highest rate recorded for the interaction of QH_2 and ferricytochrome *c* in presence of the lipoprotein alone.

TABLE V

NON-ENZYMIC REDUCTION OF CYTOCHROME *c* BY REDUCED COENZYME Q

Additions	*Duration of incubation (min)*	*mμMoles cyt. c reduced*	*Rate for 2 electron transfer/min*
Cytochrome *c* + QH_2	2	0.576	0.144
Cytochrome *c* + QH_2	14	2.83	0.101
Cytochrome *c* + QH_2 + lipoprotein	2	3.4	0.85
Cytochrome *c* + QH_2 + lipoprotein	14	10.26	0.366
Cytochrome *c* + QH_2 + lipoprotein	10	11.57	0.304

Assay conditions: 50 μmoles Tris chloride, pH 7.5; 0.5 mg ferricytochrome *c*; 700 mμmoles QH_2; 0.2 mg lipoprotein; and 0.25 *M* sucrose to 1.0 ml. The experiments were carried out at 25°.

The need for an enzyme system to catalyze the reduction of cytochrome *c* by QH_2 was further confirmed by experiments in which coenzyme Q oxidase was replaced with a highly active cytochrome *c* oxidase[17]. The preparation of cytochrome *c* oxidase used in these studies was capable of catalyzing the oxidation of about 40–45 μmoles ferrocytochrome *c*/min/mg protein. As seen in Table VI, the reduced quinone was rapidly oxidized in presence of coenzyme Q oxidase, while cytochrome oxidase was practically incapable of catalyzing this reaction. These results indicate, therefore, that coenzyme Q oxidase contains an enzyme system which catalyzes the reduction of cytochrome *c* by QH_2 and that the lipoprotein is required for the reaction. The cytochrome *c* oxidase activity of coenzyme Q oxidase has been discussed elsewhere[17]. It has been shown that coenzyme Q oxidase preparations catalyze the oxidation of cytochrome *c* in the absence of added lipoprotein at rates two to four times faster

TABLE VI

COENZYME Q OXIDASE ACTIVITY OF CYTOCHROME *c* OXIDASE

Enzyme	*Duration of incubation (min)*	*% QH_2 oxidized*
Coenzyme Q oxidase I	1	81
Coenzyme Q oxidase I	5	95.5
Coenzyme Q oxidase I	10	99.5
Cytochrome *c* oxidase	1	9
Cytochrome *c* oxidase	5	12.5
Cytochrome *c* oxidase	10	14

Assay conditions: 137 mμmoles QH_2; 0.50 mg of cytochrome *c* oxidase protein and 0.47 mg coenzyme Q oxidase protein. Additions of buffer, cytochrome *c*, and lipoprotein as in Table I. The experiments were conducted at 38°.

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than the rate at which QH_2 is oxidized. However, the presence of the lipoproteins though not mandatory as it is for the oxidation of QH_2 in the same system, increases the rate of oxidation of cytochrome *c* (ref.[17]). It may be added that this lipoprotein also stimulates the activity of highly purified preparations of cytochrome oxidase[17].

CRANE AND GLENN[11] have reported that the green fraction of ETP has DPNH oxidase activity when supplemented with cytochrome *c* but is devoid of succinoxidase activity. DPNH oxidase activity was found to be absent in preparations of coenzyme Q oxidase, which are made from the green fraction of ETP. It was found that the DPNH oxidase activity of the green fraction of ETP could be enhanced by addition of the lipoprotein to the reaction mixture, while coenzyme Q oxidase showed no DPNH oxidase activity even in the presence of both cytochrome *c* and the lipoprotein.

Components of coenzyme Q oxidase

The reduced and oxidized spectra of coenzyme Q oxidase are shown in Fig. 5. It is seen that the concentration of cytochrome *a**, as represented by the absorption peak at 605 mμ is very much higher than the concentration of the red hemoproteins.

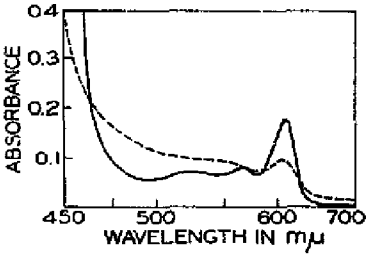

Fig. 5. The spectrum of coenzyme Q oxidase II. Protein concentration was 4.7 mg/ml in presence of 3% deoxycholate. The broken line represents oxidized spectrum and the full line after reduction with dithionite.

The small absorption bands between 570 and 500 mμ indicate the α and β bands of these hemoproteins, possibly largely of cytochrome *b*. Coenzyme Q oxidase II has about 2.9 mμmoles cytochrome *a*/mg protein and only 0.04 mμmoles/mg protein of cytochromes *b* and c_1 combined. Coenzyme Q oxidase I contains about 3.5 mμmoles cytochrome *a*/mg protein and about 0.05 mμmoles of the other cytochromes. These values were calculated from the extinction at 605 and 564 mμ of deoxycholate-solubilized preparations after reduction with dithionite. It is very interesting that the ratio of cytochrome *a* to the other hemoproteins is the same in both preparations of coenzyme Q oxidase. As was discussed earlier, coenzyme Q oxidase I is two to three times as active as preparations of coenzyme Q oxidase II on the basis of unit protein weight. The cytochrome *a* level of the two preparations also suggests that coenzyme Q oxidase I is the more purified preparation.

By the procedure described in the METHODS section, the quinone content of coenzyme Q oxidase I was estimated to be 2.94 mg/g protein and that of coenzyme Q oxidase II was found to be 2.88 mg/g protein. As was reported earlier[3], the molar concentration of coenzyme Q in mitochondria is about five times that of cytochrome *a*. In coenzyme Q oxidase I, however, there is only about 1.0 mole coenzyme Q/mole cytochrome *a*.

* In this communication no distinction will be made between cytochromes *a* and a_3.

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It was pointed out above that the method of extraction of coenzyme Q by treatment of particles with KH_2PO_4 at 90° and then extraction with cyclohexane[5] failed when applied to coenzyme Q oxidase. It was thought possible, therefore, that there might be two forms of coenzyme Q in mitochondria, one released by KH_2PO_4 and the other by saponification with alcoholic KOH. This possibility was tested by heating mitochondria in KH_2PO_4, extracting with cyclohexane, then saponifying the extracted protein and extracting again. It was found that nearly all of the coenzyme Q was removed in the first extraction. It was evident, therefore, that both methods extract almost all of the mitochondrial coenzyme Q. The lipid material removable by hot ethanol[6] was about 10 % of the total protein content in coenzyme Q oxidase I and about 6 % in oxidase II.

DISCUSSION

The studies reported here clearly indicate that coenzyme Q is situated on the substrate side of cytochrome *c*. It has been previously shown that the lipoprotein of BASFORD AND GREEN[9] contains a very high amount of bound coenzyme Q. Also, no other mitochondrial fraction which is free of the lipoprotein has yet been found to contain large amounts of coenzyme Q. Thus, it seems that this lipoprotein is the locus for mitochondrial coenzyme Q. These findings and the evidence presented in previous communications[2-4] suggest, therefore, that coenzyme Q is located in the above lipoprotein between cytochromes *b* and *c*. Cytochrome c_1 does not seem to be involved in the oxidation of coenzyme Q. It cannot replace cytochrome *c* in the coenzyme Q oxidase system and, when added in conjunction with cytochrome *c*, it does not have a stimulatory effect. Other studies* have shown that reduced cytochrome c_1 is oxidized very rapidly in a system containing catalytic amounts of cytochrome *c* and cytochrome oxidase[17]. The presence of cytochrome *c* for the oxidation of reduced cytochrome c_1 *via* cytochrome oxidase was mandatory. Thus, in an experiment where the rate of oxidation of cytochrome *c* was found to be 27.5 μmoles/min/mg enzyme protein, the corresponding rate of oxidation of cytochrome c_1 in presence of catalytic quantities of cytochrome *c* was 21.6. This rate was zero in the absence of added cytochrome *c*. These results further suggest that the pathway involving coenzyme Q is different from that involving cytochrome c_1 since the immediate electron acceptor for both coenzyme Q and cytochrome c_1 appears to be cytochrome *c*.

The finding that lipid cytochrome *c* alone can partially satisfy the requirement of the coenzyme Q oxidase system for both cytochrome *c* and the lipoprotein suggests that at least one function of the lipoprotein is to facilitate the interaction between a hydrophobic molecule (coenzyme Q) and a water-soluble one (cytochrome *c*). The stimulatory effect of the lipoprotein in the nonenzymic reduction of cytochrome *c* by reduced coenzyme Q supports this view. The inhibitory effect of antimycin A in the coenzyme Q oxidase system suggests that externally added QH_2 is oxidized by way of the coenzyme Q contained in the added lipoprotein. It was shown in previous communications[2-4] that in presence of antimycin A the reduction of bound coenzyme Q of mitochondria is inhibited. Therefore, it appears that antimycin A prevents the reduction of coenzyme Q contained in the lipoprotein. Thus, in the case of mitochon-

* Y. HATEFI, unpublished observations.

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drion-bound coenzyme Q, where this compound is situated in the lipoprotein, an inhibition of reduction was observed. While in a system such as coenzyme Q oxidase, where added QH_2 is oxidized only when the lipoprotein is present, the oxidation of *added* QH_2 (or in other words the reduction of lipoprotein-bound coenzyme Q) was inhibited by antimycin A. When mitochondria are used as catalyst for the oxidation of externally added QH_2 this process also is inhibited by antimycin A[18]. The possibility that antimycin A inhibits a process in the lipoprotein is rather appealing since the lipid nature of the latter agrees well with the solubility properties of antimycin A.

The occurrence of coenzyme Q in the lipoprotein in large concentration as well as the absolute requirement for lipoprotein in the coenzyme Q oxidase system indicates that coenzyme Q should perhaps be considered as the functional component of a more complex entity, namely the lipoprotein. This situation may be analogous to the cytochromes, wherein the hemes are the functional moieties. Thus the lipoprotein would correspond to the protein component of the cytochromes, with the difference that the former is suited for solubilizing the hydrophobic coenzyme Q molecules. The finding that cytochrome *c* can combine with the lipoprotein and assume a lipid character, the form in which it seems to react with coenzyme Q, further emphasizes the important role of the lipoprotein in the electron-transport system. It has been reported in a separate communication that this lipoprotein stimulates the activity of highly purified cytochrome *c* oxidase preparations[17]. This finding suggests that the form of cytochrome *c* which acts as a substrate for cytochrome *c* oxidase may be the same as that which is associated with the lipoprotein. Not many cases have been investigated where a lipoprotein is involved in an enzymic reaction. Another example of this nature is the thromboplastic lipoprotein which has been isolated and studied by CHARGAFF and his collaborators[19].

It was shown in a previous communication[4] that the oxido-reduction reactions of mitochondrion-bound coenzyme Q under steady-state conditions is influenced by the presence or absence of phosphate and ADP in the reaction medium. In the presence of phosphate and the absence of ADP, coenzyme Q was rapidly reduced and its rate of oxidation was remarkably decreased. Addition of ADP to the medium affected an oxidation of the reduced quinone. These findings indicated that a component of the system forms a high-energy intermediate with inorganic phosphate, which in the absence of an acceptor system such as ADP can no longer undergo cyclic reactions. As a result, electron transport ceases and the electron carriers remain in the particular state of oxidation or reduction dictated by the mechanism of oxidative phosphorylation. This state of arrested electron transport was found to accompany a shift in the oxido-reduction state of both coenzyme Q and DPN toward increased reduction. Then, when ADP was added, the high-energy phosphoryl group was presumably transferred to ADP and concomitantly coenzyme Q and DPNH were oxidized.

Recently, TODD and his associates[20] as well as HARRISON[21] have proposed that quinoid compounds may be the electron carriers which react with inorganic phosphate to form a high-energy phosphate intermediate during the process of oxidative phosphorylation. TODD and his collaborators have presented evidence that quinol phosphates can undergo dephosphorylation concomitant with oxidation. They have also shown that oxidative dephosphorylation of such compounds can result in the formation of pyrophosphate, *i.e.* phosphorylation of orthophosphate. These observations correspond rather well with the effects of phosphate and ADP on the oxido-reduction

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behavior of coenzyme Q in that in presence of phosphate the reduced form of coenzyme Q is stabilized, while oxidation of QH_2 is affected when a phosphate acceptor is available.

The possibility that coenzyme Q may form an intermediate with phosphate, which like many other high-energy phosphate compounds may be labile in aqueous media, suggests a very important role for the lipoprotein with which coenzyme Q is associated. It is possible that the lipid milieu of the lipoprotein is the necessary medium for stabilizing such an intermediate.

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