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Requirement for coenzyme Q in plasma membrane electron transport

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ABSTRACT **Coenzyme Q is required in the electron transport system of rat hepatocyte and human erythrocyte plasma membranes. Extraction of coenzyme Q from the membrane decreases NADH dehydrogenase and NADH:oxygen oxidoreductase activity. Addition of coenzyme Q to the extracted membrane restores the activity. Partial restoration of activity is also found with α-tocopherylquinone, but not with vitamin K_1. Analogs of coenzyme Q inhibit NADH dehydrogenase and oxidase activity and the inhibition is reversed by added coenzyme Q. Ferricyanide reduction by transmembrane electron transport from HeLa cells is inhibited by coenzyme Q analogs and restored with added coenzyme Q_{10}. Reduction of external ferricyanide and diferric transferrin by HeLa cells is accompanied by proton release from the cells. Inhibition of the reduction by coenzyme Q analogs also inhibits the proton release, and coenzyme Q_{10} restores the proton release activity. Trans-plasma membrane electron transport stimulates growth of serum-deficient cells, and added coenzyme Q_{10} increases growth of HeLa (human adenocarcinoma) and BALB/3T3 (mouse fibroblast) cells. The evidence is consistent with a function for coenzyme Q in a trans-plasma membrane electron transport system which influences cell growth.**

Coenzyme Q (CoQ, ubiquinone) is present in the endomembranes of cells (1–4) as well as in mitochondria, where it serves as a central component of the transmembrane electron transport system (5). The CoQ in endomembranes is concentrated in the Golgi apparatus and plasma membrane (4). The high concentrations of CoQ in these membranes raise the question of its function within the extramitochondrial membranes. Possible functions include storage for transfer to mitochondria or the blood serum (4, 6), action as a renewable antioxidant within the lipid bilayers (7, 8), or function as an electron carrier in these membranes (3). Evidence for a role in electron transport includes reduction of the CoQ in microsomes with NADH and reoxidation by ferricyanide (9, 10).

Plasma membranes also contain oxidoreductase enzymes (11), including a trans-plasma membrane electron transport system that influences the growth of cells (12), activates phosphorylation of membrane proteins (13), and induces expression of c-*myc* and c-*fos* protooncogenes (11). Evidence is presented that CoQ functions in transmembrane electron transport and that added CoQ stimulates growth of HeLa and BALB/3T3 cells in the absence of serum.

EXPERIMENTAL PROCEDURES

Cell Culture. HeLa cells were grown on α minimal essential medium in a 5% CO_2 atmosphere at 37°C, pH 7.4. Unless otherwise indicated, 10% fetal bovine serum, 100 units of penicillin per ml, and 170 μg of streptomycin per ml were added. Cells for assay were released by mild trypsinization and centrifuged at 150 × *g* for 7 min, and the pellet was taken up in TD buffer [0.14 M NaCl/5 mM KCl/0.7 mM Na_2HPO_4/25 mM Trizma base (Sigma), pH 7.4] to a final concentration of 0.1 g of cell weight per ml (14).

Plasma Membrane Preparation. The two-phase separation procedure adapted for rat liver plasma membranes was used (15). Isolated membranes were characterized by marker enzymes and by morphometry. Human erythrocyte membranes were prepared from blood bank erythrocytes (16) with final separation on a dextran gradient.

Trans-plasma Membrane Electron Transport from Cells. Ferricyanide reduction by HeLa cells was measured by decrease of absorbance at 420 nm in the supernatant after removal of cells (17). The reaction mixture contained TD buffer, 0.01–0.05 g (wet weight) of cells, and 0.1 mM ferricyanide. The reaction was stopped in an ice bath. Controls with 2 μM rotenone showed no mitochondrial release from broken cells (17). An alternative procedure was measurement of ferricyanide reduction by absorbance change at 410 nm minus 500 nm in the dual-beam mode of a spectrophotometer (14).

Membrane Dehydrogenase and Oxidase Assays. NADH ferricyanide reductase was measured in 2.8 ml of 50 mM Tris chloride buffer (pH 7.4) with 25 μM NADH, 0.1 mM potassium ferricyanide, and 20–80 μg of plasma membrane by following the decrease in absorbance at 420 minus 500 nm with the dual-beam mode of the Aminco DW2a spectrophotometer at 37°C. The ferricyanide extinction coefficient used was 1.0 $mM^{-1} \cdot cm^{-1}$. Boiled plasma membrane was a control for the nonenzymatic chemical reaction between NADH and ferricyanide (18). NADH diferric transferrin reductase was assayed in the same buffer, using 15 μM NADH and 10 μM diferric transferrin (19). Release of ferrous iron was measured by formation of ferrous bathophenanthroline disulfonate as 535 minus 600 nm absorbance change, using the dual beam with an extinction coefficient of 17.6 $mM^{-1} \cdot cm^{-1}$ at these wavelengths. Controls were without enzyme or without NADH. NADH oxidase was measured by the decrease in absorbance at 340 minus 430 nm in the dual beam with an extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$. The reaction mixture contained 15 μM NADH, 1 mM KCN, and 20–80 μg of membrane in 2.8 ml of 50 mM Tris chloride (pH 7.4). Additions were preincubated with the membrane for 3–5 min before the reaction was started with NADH. Control assays were without membrane (19).

NADH CoQ_{10} Reductase. The reduction of CoQ_{10} added to the liver plasma membrane by evaporation of a solution in heptane on the lyophilized membrane was followed by absorbance change at 410 minus 500 nm with an extinction coefficient of 0.7 $mM^{-1} \cdot cm^{-1}$ (20). Total CoQ_{10} in the mem-

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Abbreviations: CoQ, coenzyme Q; αTQ, α-tocopherylquinone; DClQ, 2,3-dimethoxy-5-chloro-6-naphthylmercapto-1,4-benzoquinone; ETHOXQ, 2-methoxy-3-ethoxy-5-methyl-6-hexadecylmercapto-1,4-benzoquinone.

brane was measured by borohydride reduction of an aliquot of CoQ_{10}-supplemented membrane.

Oxidant-Induced Proton Release from Cells. Proton release was measured as acidification of the suspension medium [3 ml of 150 mM NaCl in a thermostatted cuvette with 1.5 mM Tris chloride (pH 7.4) to dampen pH oscillation] (21). The mixture was stirred and was bubbled continuously with air to remove excess CO_2. A Corning combination electrode measured pH change between pH 7.4 and 7.0. After equilibration, 0.1 mM ferricyanide or 10 μM diferric transferrin was added to initiate proton release. Calibration was with 50 nmol of standard HCl at the beginning and end of each assay (21). Cells were incubated with CoQ analogs for 3–5 min before the pH equilibration was started.

RESULTS

Extraction and Restoration. Extraction of lyophilized rat liver plasma membrane for 4–6 hr at 20°C in the dark removed 60–80% of the total CoQ in the membrane (Table 1).

The NADH ferricyanide reductase activity decreased to 20–40% of the activity in the unextracted lyophilized membrane (Table 1). CoQ_{10} was added to the extracted membrane in heptane, and the heptane was removed by evaporation (22). The extracted membranes with added CoQ or α-tocopherylquinone (αTQ) showed a partial restoration of NADH ferricyanide reductase (Table 1). Addition of the quinones in ethanol to extracted membranes in assay buffer, followed by 3–5 min of incubation, also partially restored NADH ferricyanide reductase, but not as well as addition in dry heptane (data not shown). CoQ did not increase activity in unextracted membranes. Vitamin K_1 inhibited NADH ferricyanide reductase in both untreated and extracted membranes (Table 1).

The diferric transferrin-stimulated NADH oxidase of rat liver plasma membranes also was decreased by extraction with heptane. CoQ partially restored both the cyanide-insensitive NADH oxidase and the diferric transferrin stimulation (Table 2). Addition of CoQ_{10} to unextracted membrane slightly increased diferric transferrin-stimulated NADH oxidase activity.

Extraction of human erythrocyte membranes with heptane inhibited NADH ferricyanide reductase as much as 80% (Table 1). Addition of CoQ_{10} in ethanol to the membranes in assay medium partially restored the NADH ferricyanide reductase. αTQ also restored some activity, but vitamin K_1 was ineffective (Table 1).

Table 1. Restoration of NADH ferricyanide reductase activity to rat liver and erythrocyte plasma membranes with quinones after heptane extraction

Membrane treatment	Activity, nmol/min per mg of protein	
	Liver	Erythrocyte
Control	216 ± 18 (3)	318 ± 39 (3)
+ 10 μM CoQ_{10}	220	
Extracted	59	60 ± 13 (3)
+ 10 μM CoQ_{10}	107	265 (2)
+ 10 μM αTQ	99	135 (2)
+ 15 μM vitamin K_1	16	27 (2)

Lyophilized rat liver membranes (19 mg of protein) was extracted with 15 ml of heptane for 6 hr at 20°C in the dark. Heptane was decanted and evaporated. Quinones were added back to the membrane in 5 ml of heptane. The heptane was removed by evaporation and the membranes were taken up in 50 mM Tris chloride (pH 7.4) for assay. Original CoQ in the membrane was 20.9 nmol, and 16.2 nmol was recovered in the heptane extract. CoQ does not stimulate ferricyanide reduction in lyophilized unextracted membrane. Representative results [mean ± SD (n)] are shown for five experiments with CoQ_{10}. For erythrocyte membranes, quinones were added in ethanol to membranes in buffer 4 min before NADH.

Table 2. Restoration of ferric transferrin-stimulated NADH oxidase of heptane-extracted rat liver plasma membrane by CoQ_{10}

Membrane treatment	NADH oxidase, nmol/min per mg of protein	
	Unstimulated	Transferrin-stimulated
Control	7.7 (2)	10.0 (2)
+ 10 μM CoQ_{10}	8.2	12.6
Extracted	1.2 (2)	1.8 (2)
+ 10 μM CoQ_{10}	5.5 (2)	7.9 (2)

Extraction was as described for Table 1 but for 7 hr. Assay was as described, with 10 μM ferric transferrin added after 10 min (19). Similar effects were observed in two other experiments.

Analog Inhibitions. 2,3-Dimethoxy-5-chloro-6-naphthylmercapto-1,4-benzoquinone (DClQ) and 2-methoxy-3-ethoxy-5-methyl-6-hexadecylmercapto-1,4-benzoquinone (ETHOXQ) inhibited NADH ferricyanide reductase of rat liver plasma membranes, and the inhibition was reversed partially with CoQ (Table 3). Chloroquine (23) also showed a partial reversal of inhibition in the presence of CoQ_{10}.

The NADH oxidase and the diferric transferrin-stimulated NADH oxidase of rat liver plasma membrane were inhibited 100% by DClQ (24 μg/ml) or ETHOXQ (25 μg/ml). Piericidin also inhibited the oxidase activity, and the inhibition was partially reversed by CoQ (3). Capsiacin inhibited the diferric transferrin-stimulated NADH oxidase activity 88% at 150 μM, and the inhibition was only 15% with 10 μM CoQ_{10}.

DClQ and ETHOXQ also inhibited the NADH ferricyanide reductase of human erythrocyte plasma membranes (Table 3). Both CoQ and αTQ partially restored activity, whereas vitamin K_1 tended to be inhibitory.

Ferricyanide reduction by HeLa cells was also inhibited by DClQ (Fig. 1), and the inhibition was partially reversed by CoQ. ETHOXQ gave 70% inhibition at 24 μg/ml, and this inhibition was completely reversed by CoQ_{10} (Fig. 1). Piericidin at 0.1 μM inhibited ferricyanide reduction by HeLa cells 72%. Capsiacin inhibits ferric ammonium citrate reduction by HeLa cells (24) 40% at 100 μM and 87% at 200 μM.

CoQ Reductase Activity. The reduction of CoQ can be measured by a decrease in absorbance at 410 nm (extinction coefficient, 0.7 $mM^{-1} \cdot cm^{-1}$). When the absorbance of rat liver plasma membrane was measured at 410 minus 500 nm when NADH was added, there was a slight decrease in absorbance (Fig. 2*A*). If a 20-fold excess of CoQ was added

Table 3. Effect of CoQ analogs on NADH ferricyanide reductase of erythrocyte and rat liver plasma membranes

Addition(s)	NADH ferricyanide reductase	
	Erythrocyte, nmol/min per mg of protein	Liver, % control
None	308 ± 43	100
DClQ (25 μg/ml)	113 ± 37	47
+ αTQ (10 μM)	275 ± 7	
+ CoQ_{10} (10 μM)	215 ± 26	91
+ vitamin K_1 (15 μM)	71 ± 12	61
ETHOXQ (30 μg/ml)	30 (2)	50
+ αTQ (10 μM)	239 ± 20	
+ CoQ_{10} (10 μM)	232 ± 19	84
+ vitamin K_1 (15 μM)	100 ± 21	
Chloroquine (0.5 mM)		17
+ CoQ_{10} (10 μM)		45

Inhibitors and quinones were incubated with membrane for 3 min before assay. Standard deviations were based on triplicate assays using the same membrane preparation. Control (100%) rates for rat liver plasma membrane for chloroquine and DClQ, 72.6; for ETHOXQ, 199 nmol/min per mg of protein.

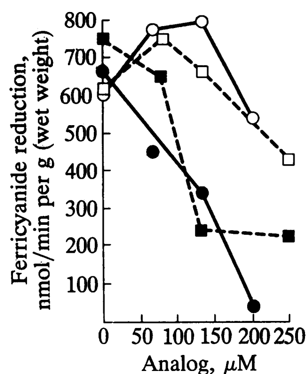

FIG. 1. Inhibition (filled symbols) of ferricyanide reduction by HeLa cells with DClQ and with ETHOXQ and reversal of inhibition (open symbols) by CoQ_{10} (10 μM). Circles, DClQ; squares, ETHOXQ.

to the membrane, then NADH caused a much greater decrease in absorbance at 410 nm, observed whether NADH was added last (Fig. 2*B*) or CoQ-supplemented membrane was added last (Fig. 2*C*). This absorbance change is consistent with the presence of an NADH CoQ reductase. When the CoQ analog ETHOXQ was added at 30 μg/ml, less absorbance decrease at 410 nm occurred (Fig. 2*D*), showing inhibition of the NADH CoQ reductase. On the other hand, 1 μM rotenone did not inhibit the CoQ reduction. Measurement of the NADH CoQ reductase by oxidation of NADH with rat liver plasma membrane gave unclear results because rat liver plasma membrane has NADH oxidase activity, and we do not have good inhibitors for the oxidase part of that activity. With erythrocyte plasma membranes, which have no NADH oxidase activity (25), NADH decreased when CoQ was added (Table 4). The activity observed could represent NADH CoQ reductase activity or the activation of NADH oxidase activity in the erythrocyte membrane.

Effects of CoQ on Cell Growth. The growth of HeLa cells in serum-free medium was more than doubled with high levels of CoQ (Fig. 3). Ferricyanide gave added stimulation. The effect was observed in nine separate experiments, and the growth stimulation at 20–30 μM CoQ in those experiments averaged 125 ± 35%. Growth of BALB/3T3 cells increased 100% at 30 μM CoQ and 63% at 10 μM CoQ in serum-free medium in 48 hr. An increase from 1.5×10^5 to 3.0×10^5 cells per 25^2-cm flask was observed in 48 hr.

Table 4. NADH CoQ_{10} reductase activity with human erythrocyte plasma membranes

Additions	NADH oxidation, nmol/min per mg of protein
Membrane + NADH	0
Membrane + CoQ_{10} (5 μM) + NADH	3.3
Membrane + CoQ_{10} (10 μM) + NADH	5.4
Membrane + NADH, follow with CoQ_{10} (10 μM)	2.7

Assay was in 3.0 ml of 50 mM Tris chloride (pH 7.4) with 25 μM NADH and 0.24 mg of erythrocyte membrane protein. NADH oxidation was measured at 340 nm minus 430 nm. Membrane was incubated with CoQ_{10} for 3 min before assay when CoQ_{10} was added first. When CoQ_{10} was added after the membrane and NADH there was a 3-min lag before the full oxidation rate was reached. CoQ_{10} was added in ethanol. Ten microliters of ethanol alone gave no activity. Two similar experiments repeated this effect.

HeLa cell growth also was stimulated 65% at 20 μM αTQ in the absence of ferricyanide (Fig. 3).

Inhibition of Diferric Transferrin-Stimulated Proton Release by HeLa Cells. CoQ analogs inhibit the proton release by HeLa cells stimulated by diferric transferrin (21). The inhibition was reversed by 10 μM CoQ. The same amount of CoQ alone caused a slight stimulation of proton release (Table 5).

DISCUSSION

Plasma membranes of eukaryotic cells have enzymes that transfer electrons from internal NADH to external electron acceptors (11, 12). The presence of CoQ in plasma membranes (4) allows its consideration as an electron carrier. CoQ quinol at 26–37% in plasma membranes is consistent with a role in oxidation–reduction reactions (26). Since coenzyme Q in blood is mostly in quinol form (27), the plasma membrane electron transport may supply electrons for reduction.

Additional evidence for CoQ function in trans-plasma membrane electron transport is presented here. Extraction of CoQ from the membrane decreases NADH dehydrogenase activity, and added CoQ_{10} partially restores activity. CoQ analogs inhibit NADH dehydrogenase in plasma membranes, reduction of external oxidants by whole cells, and oxidant-induced proton release as well. The analog effects are reversed by CoQ_{10}. In addition, NADH CoQ reductase activity can be demonstrated with isolated plasma membranes.

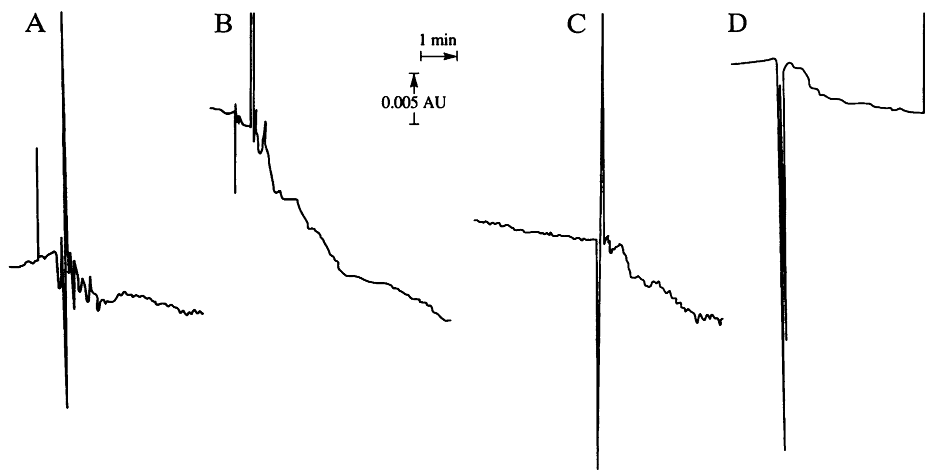

FIG. 2. Demonstration of NADH CoQ reductase activity in rat liver membrane with added CoQ_{10}, and inhibition of reduction by ETHOXQ in 50 mM Tris chloride (pH 7.4) in 2.8-ml total volume. Absorbance (AU) was measured at 410 minus 500 nm. Times of additions are indicated by large deflections. (*A*) NADH (50 μM) with 0.42 mg of membrane protein added. (*B*) CoQ-reconstituted membrane (0.42 mg) with 50 μM NADH added. (*C*) NADH (50 μM) with 0.42 mg of reconstituted membrane added. (*D*) NADH (50 μM) and ETHOXQ (30 μg/ml) with 0.42 mg of reconstituted membrane added.

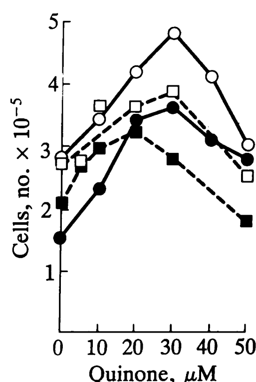

FIG. 3. Stimulation of HeLa cell proliferation by CoQ_{10} in serum-free medium with (open symbols) and without (filled symbols) ferricyanide (0.01 mM). Results are representative of nine separate experiments with 10–30 μM CoQ. A decrease is not always observed at 40–50 μM CoQ. Equivalent volumes of added ethanol gave only slight growth inhibition. For αTQ in a separate experiment, 30 μM gave 86% and 15 μM gave 53% increase in cell count. Circles, CoQ_{10}, squares, αTQ.

The basis for the response to CoQ is not mitochondrial contamination. The liver plasma membranes prepared by the aqueous two-phase partition have <2% mitochondria (15). The human erythrocyte membranes are free of mitochondria as judged from marker enzyme assays (16). Further, the response of NADH ferricyanide reductase to changes in CoQ is not the same in plasma membranes as in mitochondria.

Pentane extraction of lyophilized membranes is the best method to demonstrate a CoQ requirement for NADH oxidase activity in mitochondria (22). NADH ferricyanide reductase activity of mitochondria does not require CoQ. In contrast, both the NADH ferricyanide reductase and the ligand-activated NADH oxidase in the plasma membrane are decreased when CoQ is removed, and they can be partly restored by added CoQ. Activity is best restored by addition of CoQ in heptane to the lyophilized extracted membranes, followed by evaporation of the heptane (22). Addition of CoQ_{10} in ethanol to the extracted membranes suspended in assay medium also restores activity.

The ligand-activated NADH oxidase of rat liver plasma membrane is different from the NADH oxidase of mitochondria in that (*i*) it is activated by specific ligands for plasma membrane receptors such as diferric transferrin, growth factors, or triiodothyronine, (*ii*) it is not stimulated by cytochrome *c*, and (*iii*) it is not inhibited by cyanide, azide, or rotenone (12, 28). The oxidase retains these properties after solvent extraction when reconstituted with CoQ.

A surprising result is the effectiveness of αTQ in restoration of both NADH ferricyanide reductase and NADH oxidase in the extracted plasma membrane. αTQ also reverses inhibition of NADH ferricyanide reduction by CoQ analogs. αTQ is ineffective for restoration of activity in extracted mitochondria (29). The lack of restoration by vitamin K_1 is evidence for specificity in the quinone requirement.

Table 5. Inhibition of diferric transferrin-stimulated proton release from HeLa cells by CoQ analogs

	nmol/min per g (wet weight)	
Addition(s)	H^+ release with transferrin	Ferricyanide reduction
None	686 ± 32 (3)	740
DCIQ (25 μg/ml)	140	320
DCIQ + CoQ_{10} (10 μM)	1068	660
ETHOXQ (15 μg/ml)	307	510
ETHOXQ + CoQ_{10}	727	800
CoQ_{10}	856	600

Diferric transferrin (10 μM) was used to stimulate proton release (21) and 0.1 mM ferricyanide was used for the reduction assay. CoQ_{10} (10 μM) was added with inhibitor. Ferricyanide-induced proton release was inhibited 40% and ferricyanide reduction was inhibited 46% at 0.1 μM piericidin A.

αTQ is not found in fresh mitochondria, but both liver and erythrocyte plasma membranes contain a significant amount. Human erythrocyte plasma membranes have more αTQ (1.2 nmol/mg of protein) than CoQ (0.01 nmol/mg of protein), which may relate to the lack of NADH oxidase activity in erythrocyte membranes (25).

The use of quinone analogs for inhibition of electron transport in both mitochondria and chloroplasts has been rationalized on the basis of binding at specific quinone binding sites in the electron transport complexes. Some of the best inhibitors of mitochondrial electron transport, such as rotenone and antimycin A, act at CoQ binding sites of complex I, II, or III in the mitochondria (30) but are ineffective as inhibitors of plasma membrane electron transport. Piericidin A inhibits mitochondrial NADH oxidase activity but does not inhibit mitochondrial NADH ferricyanide reductase activity (31). In plasma membrane it inhibits both NADH oxidase and NADH ferricyanide reductase activity, and the inhibition can be partially reversed by CoQ. Chloroquine (32) and capsiacin (33) also inhibit CoQ function in mitochondria, and these compounds show CoQ-reversible inhibition of NADH oxidase and NADH ferricyanide reductase in plasma membranes. ETHOXQ and DCIQ are CoQ analogs identified as reversible inhibitors of NADH ferricyanide reductase and NADH oxidase of plasma membranes.

Analog inhibition allows study of quinone function in ferricyanide reduction by intact cells, where the extraction of quinones with organic solvents is not feasible. Ferricyanide reduction occurs at the cell surface, and so effects on CoQ sites in mitochondria would not inhibit the activity. Inhibitors of mitochondrial electron transport such as cyanide, antimycin A, or rotenone do not inhibit trans-plasma membrane electron transport in liver (17) or HeLa cells (18). Piericidin, DCIQ, and ETHOXQ inhibit ferricyanide reduction by HeLa cells, and the inhibition is reversed by CoQ_{10}.

Ferricyanide reduction or diferric transferrin activation of the oxidase activates proton release through Na^+/H^+ exchange (21). The CoQ analogs DCIQ or ETHOXQ inhibit both ferricyanide- and diferric transferrin-stimulated proton release, and this inhibition is reversed with CoQ.

The components of the trans-plasma membrane electron transport chain have not been elucidated. A plasma membrane NADH oxidase has major polypeptides of 34 and 72 kDa (28). A transmembrane dehydrogenase from yeast has been cloned and sequenced. It is a polypeptide of 68 kDa with six hydrophobic helix regions, which may represent transmembrane segments (34). CoQ and αTQ, as well as flavin, thiols, cytochrome *b*-type hemes, iron, and copper, have been identified in some plasma membranes (12, 25).

The stimulation of cell proliferation in serum-free medium by external oxidants such as ferricyanide has been observed with many different cell lines (11, 12). The natural electron acceptor for growth stimulation can be oxygen, through the transferrin-stimulated NADH oxidase in the plasma membrane (28, 35).

CoQ stimulates growth of HeLa and BALB/3T3 cells in serum-free medium both in the presence and in the absence of ferricyanide. On the other hand, ferricyanide reduction by isolated liver plasma membrane is clearly not stimulated by added CoQ. The NADH oxidase may be stimulated slightly by CoQ. Proton release by HeLa cells is also enhanced by CoQ. Since the effects of CoQ and ferricyanide on growth stimulation are additive, the mechanisms for stimulation of growth appear to be different.

The basis for the oxidant and CoQ stimulation of cell growth remains to be established. The redox system has been shown to activate messenger systems such as changes in internal pH (35), calcium ions (36), the NADH/NAD ratio (37), membrane potential, and protein phosphorylation (13).

The CoQ content of human serum ranges from 0.7 to 1.2 μM, and it is associated primarily with the very low density lipoproteins (38). This concentration is below the levels we use to stimulate growth, but the carrier system may be important to make CoQ available to cells. Addition as an ethanolic solution to aqueous medium is nonphysiological. Association of lipophilic quinones with protein in blood may facilitate growth at lower quinone concentrations.

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