COENZYME Q:

Molecular Mechanisms in Health and Disease

Edited by
Valerian E. Kagan
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Preface

Since its discovery in 1957 by Crane and associates, coenzyme Q (CoQ, ubiquinone Q_{10}) has become the subject of extensive studies in bioenergetics. This resulted in the discovery of its unique and remarkable role in energy production in mitochondria. The ubiquitous presence of CoQ in essentially all types of intracellular membranes and lipoproteins suggests that there are additional roles for CoQ in cellular biochemical pathways associated with its redox properties. One such role is that, in its reduced form, CoQ acts as an electron donor to reduce reactive chain-initiating and chain-propagating radicals, i.e., functions as a radical scavenger or chain-breaking antioxidant. Later findings implicated CoQ in extramitochondrial electron-transport systems, demonstrating its utility as a universal redox component. Since a variety of biological functions depend on CoQ, the question of deficiency or dysregulation leading to pathological states can be raised. Because of this, an emphasis was put on tissue levels of CoQ in maintaining health and its possible roles in disease.

This volume attempts to summarize the latest developments in these very different areas of CoQ research. It covers a broad spectrum of different fields in which CoQ represents a subject of investigation—from physical chemistry and biophysics through biochemistry, molecular biology, and cell biology to nutritional sciences, medical applications, and geriatrics. Not surprisingly, levels and depth of mechanistic understanding and description of CoQ's different effects are radically different in these different fields and this is reflected in the chapters into which this book has been divided.

Another specific feature of the book is the widespread geography of contributing authors, with all corners of the globe being represented. This reflects not only a wide-ranging geographic interest in research on CoQ, but also provides a number of the heterogenous approaches and styles reflected in the chapters in the volume. An international team of experts joined forces to produce a work that addresses the major facets of CoQ research and creates a must-have resource for researchers in the field. With this understanding, the editors did not attempt to make the volume more uniform at the expense of losing the bright and colorful originality furnished by selected contributors—all experts in their respective fields. Instead, the editors believed that the most essential goal is a balanced approach encompassing the most important achievements in the field.

Hands-on experts describe in detail the key findings, discoveries, and concepts in different aspects of CoQ research. Therefore, the reader will find chapters describing the topography and behavior of CoQ in membranes (P. Quinn and G. Lenaz) as well as its most essential antioxidant chemical properties (K. Mukai). This is followed by wonderfully presented contemporary ideas on the bioenergetic mechanisms for CoQ in mitochondria (P.L. Dutton et al.). New concepts on extramitochondrial functions of CoQ (in plasma membranes and lysosomes) are discussed in two chapters (P. Navas et al. and H. Nohl and L. Gille). Recent discoveries in biosyntheic pathways for CoQ based on molecular genetic approaches are presented in the chapter by C. Clarke and T. Jonassen. Several chapters are dedicated to detailed descriptions of the antioxidant mechanisms of CoQ in membranes. These include considerations of antioxidant dynamics of CoQ in membranes (E. Niki), its special role in antioxidant protection of lipoproteins (S.R. Thomas and R. Stocker), and its antioxidant interactions with vitamin E (V. Kagan et al.). The chapter by H. Nohl et al. demonstrates that, despite an almost unequivocally accepted antioxidant role for CoQ, there is still enough room for further research as it identifies conditions under which CoQ may become a source of reactive oxygen species rather than their scavenger. A special chapter is focused on biochemical and pharmacological properties of CoQ analogs (A. Mordente et al.)

As a transition to the health effects of CoQ, C. Weber reviews issues related to dietary intake and sources of CoQ. Important methodological information on assays and handling of samples for CoQ analysis can be obtained in the chapter presented by Rousseau et al.. Additionally, three chapters are focused on plasma levels of CoQ as potential markers of abnormal status of the organism, i.e., disease. In fact, chapters by A. Kontush, Y. Yamamoto and S. Yamashita, and J.B. Ubbink describe the potential use of CoQ measurements as diagnostic predictors of disease.

Several chapters discuss health effects of CoQ in experimental conditions (animal studies) or in clinical settings (chapters by D. Das and H. Otani, Alho et al., A. Gvozdjakova and K. Jarmila, and G.P. Littaru and M. Battino). The role of CoQ in liver diseases is presented in two chapters discussing alcohol-induced liver injury (S. Eaton et al.) and liver carcinogenesis (P. Stal and J.M. Olsson).

Special attention was paid to an issue of potential benefits that CoQ supplementation may offer in sport and physical exercise (C. Malm and M. Svensson, T.J. Vasankari and Ahotupa, and J. Faff). Finally, relationships between CoQ and longevity are the subject of the chapter written by H. Alho and K. Lonnrot.

While studies of CoQ mechanisms in mitochondrial energy production have been recognized by Peter Mitchell's 1978 Nobel Prize for chemistry, many essential mechanistic details have become more evident now. Some of the earlier outstanding contributors to the subject, such as Karl Folkers and Lars Ernster, have now left the field to be replaced by new talents striving to discover and learn about other functions of CoQ. It is this still-incomplete knowledge of biosynthesis, transport, delivery, biochemical pathways, and pathological disregulation of CoQ that limits its effective use in health and disease. We hope that the summary of CoQ research contained in this volume will contribute to furthering our understanding of its role and functions and stimulate further research critical for future applications.

Valerian Kagan and Peter Quinn

About the Editors

Valerian E. Kagan received his Ph.D. degree in biochemistry and biophysics from M.V. Lomonosov Moscow State University, and his D.Sc. degree from the USSR Academy of Sciences, Moscow. In 1983, Dr. Kagan was awarded the State Prize of the USSR for science. From 1976 through 1983, Dr. Kagan was an associate research professor at M.V. Lomonosov Moscow State University in Moscow, and from 1983 to 1989 he was a research professor and head of the Membrane Biostabilization Group in the Institute of Physiology Sofia (Bulgaria). Dr. Kagan was an associate research biochemist in the Department of Molecular and Cell Biology at the University of California, Berkeley, and a visiting scientist at Lawrence Berkeley Laboratory. Since 1992, Dr. Kagan has been an associate professor of environmental and occupational health, and of pharmacology at the University of Pittsburgh. He is also a member of the University of Pittsburgh Cancer Institute and holds a visiting professorship at King's College, London.

Dr. Kagan's research interests are focused on free radicals and antioxidants in biology and medicine, genotoxicity of free radicals, oxidative stress mechanisms in apoptosis, and biochemistry of nitric oxide. He has published more than 300 papers in peer-reviewed journals.

Peter J. Quinn, Ph.D., is currently professor of biochemistry at King's College, London, where he heads a research team using a range of biophysical methods to investigate the structure of biological membranes and their constituents. One of Dr. Quinn's particular interests is in the interaction of coenzyme Q and vitamin E with the membrane lipid matrix. His undergraduate degree was obtained from the University of Melbourne, and his postgraduate degrees from the University of Sidney and London University. He holds a visiting professorship at the University of Pittsburgh and Robert Gordon University.

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1 Mobility of Coenzyme Q in Membranes

Giorgio Lenaz

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1.1 INTRODUCTION

Coenzyme Q(Q) or ubiquinone was discovered as a mobile substrate-like component of the mitochondrial respiratory chain:[1] hence, its mobility has received much attention both on biochemical and biophysical grounds. On the other hand, novel functions discovered for Q also require knowledge of its mobility properties. For example it is not known if its role in plasma membrane electron transfer[2] requires the quinone as a mobile intermediate; its membrane-bound antioxidant function[3,4] may require its high mobility within the lipid bilayer, e.g., for its possible interaction with vitamin E.[5] This review deals with the present knowledge of the role of mobility, in particular lateral diffusion, in the function of Q, largely derived from studies on mitochondrial electron transfer. Therefore, it seems appropriate to present an introductory chapter on the physical bases of mobility of membrane-bound molecules.

1.2 MOBILITY OF MEMBRANE COMPONENTS

The mobility of membrane-bound molecules is essential for many biological functions.[6] Although all membrane molecules exist in a dynamic state, fluidity is not distributed homogeneously but varies within lateral domains in the plane of the bilayer.[7] It was suggested, however,[6] that the

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organizational problem in most membranes is not to maximize motion of all their component molecules, but to control this motion with specific restrictions.

1.2.1 Lateral Diffusion

Mobility of membrane components includes both their lateral bidimensional displacement in the plane of the membrane (*lateral diffusion*) characterized by a lateral diffusion coefficient D_l, and rotational motion about an axis perpendicular to the plane of the membrane (*rotational diffusion*), characterized by a rotational diffusion coefficient D_r.

Brownian motion or diffusion is the random movement of a particle due to exchange of thermal energy with its environment, so that both its position and its orientation exhibit noise. A rigid object in space has three positional and three angular coordinates, each being a randomly fluctuating function of time. For a spherical particle in slow motion in a three-dimensional viscous fluid we have:

$$D_l = kT/6\pi\eta R \tag{1.1}$$

$$D_r = kT/8\pi\eta R^3 \tag{1.2}$$

where k is Boltzmann's constant, T is the absolute temperature, η is the viscosity, and R is the particle radius.

In biological membranes, treatment of the corresponding equations is complicated by the reduction of dimensionality and by the anisotropic nature of the medium (the lipid bilayer). A hydrodynamic model of membrane diffusion was given by Saffman and Delbrück[8] for a cylindrical object embedded in a viscous continuum fluid sheet bounded by an aqueous fluid. Such a particle, simulating a membrane protein, is restricted to moving laterally in the x-y plane and to rotate around the z axis. Assuming the viscosity of the membrane η is much higher than the viscosity of the outer medium η', the following equations apply:

$$D_{\mathrm{r}} = kT/4\pi\eta a^2 h \tag{1.3}$$

$$D_l = (kT/4\pi\eta h)\,(\log\, \eta h/\eta' a - \gamma) \tag{1.4}$$

where h is the height of the cylinder, a is its radius, and γ is Euler's constant (0.5772). The model assumes that the viscosity of the fluid bathing the membrane in addition to that of the lipid phase itself affect protein lateral diffusion.

The lateral diffusion of lipids and lipid-like molecules and of hydrophobic molecules embedded in the lipid bilayer, however, are not expected to obey the Saffman-Delbrück model by depending on the viscous drag from the outer medium. In the case of a diffusant comparable in size with the solvent, *the free volume theory*[9] applies best.[10] According to this theory, the diffusion of a molecule in a fluid system may be divided into a three-step process: (i) creation of local free volume by density fluctuations that open a hole within the cage where a solute molecule is situated; (ii) the jump of the diffusing molecule into this hole, creating a void at the previous position; (iii) the filling of the void by another solvent molecule. The lateral mobility of amphipathic molecules in a fluid lipid matrix will be determined by the free area according to

$$D_l = A\,\exp(\delta a^*/a_{g(T)}) \tag{1.5}$$

where a^* is the close packed area per molecule and $a_{g(T)}$ is the mean free area per molecule at a given temperature T, and δ is a constant; the pre-exponential factor A is related to a^* and the gas kinetic velocity of the diffusant.

It is expected that the lateral mobility of amphipathic molecules is determined by the free outer polar region of the bilayer, whereas nonpolar molecules diffuse according to the freedom of the central region, which experiences much greater fluidity[11] with a higher expected probability to form void volumes. Berg[12] provided a series of equations relating viscosity and molecular dimensions for molecules of different shapes moving in two dimensions.

The method of choice for measuring lateral diffusion of proteins in membranes is *fluorescence recovery after photobleaching (FRAP)*;[13] the method can be applied to nonchromophoric proteins by attaching suitable covalent probes. FRAP involves photochemical bleaching of the chromophore in a small region of the membrane with a strong pulse of laser excitation; as the unbleached molecules diffuse into the bleached areas, the fluorescence intensity of the area increases; D_l are calculated from the recovery curves. The disadvantage of chemical modification of the diffusing molecule may be of major importance for small molecules.

A limitation of the method is that D_l measured by FRAP in nonplanar membrane surfaces, as is often the case in membranes with microvilli or invaginations, is underestimated, representing the mobility in projected flat planes.[14] The method is unsuitable for membranes of small diameter, like subcellular organelles, unless they are modified to increase their size by fusion or other means.[15] The possibility that membranes are laterally heterogeneous over short distances[16] makes FRAP only suitable for measuring long-range (>1 μm) lateral diffusion. Since collision-dependent interactions are more directly related to local rather than to long-range diffusion,[17] FRAP may not be suitable for determining the role of diffusion in collisional processes.

Other techniques are used to study short-range lateral diffusion, but appear to be unfit for protein diffusion. Such techniques include, among others, esr line broadening of spin labels,[18] NMR,[19] pyrene excimer formation,[10] and fluorescence collisional quenching.[20] All these methods involve the determination of the rate of collision encounters between two molecules, and therefore can only measure local short-range diffusion, as is involved in chemical reactions and molecular diffusion-dependent associations.[21]

The D_l of lipids, investigated by a variety of methods, usually range between 10^{-7} and 10^{-8} cm^2/s.[22] They appear to fit the free-area theory and are slightly affected by the density of proteins in the membrane. Contrary to lipids, the D_l of membrane proteins usually range in a broader field,[13] between $>10^{-9}$ cm^2/s and complete immobilization. Protein diffusion in model lipid bilayers obeys the Saffman-Delbrück model;[23] in natural membranes, however, diffusion is slower than theoretically expected,[13] suggesting that mobility is hindered by a number of physiological restrictions.

One possible restriction is the increase of membrane viscosity:[24] D_l of proteins are usually decreased by 2–3 orders of magnitude below the lipid phase transition.[25] However, the correspondence between membrane viscosity and protein diffusion is often poor, suggesting that lateral mobility *in situ* is mainly modulated by constraints from the aqueous matrix. The elements of the cytoskeleton,[26] in particular the microfilaments or other peripheral proteins, produce a large reduction of the measured D_l. Other restrictions are represented by membrane junctions.

A factor strongly modifying protein diffusion is their concentration in the membrane. The dependence of the lateral distribution of membrane proteins on the protein lipid ratio has been modeled by Monte-Carlo calculations[27] and shown to vary from random to aggregated in a continuous network. It was calculated that long-range diffusion is relatively sensitive to the area fraction of the membrane proteins; at a critical area fraction, diffusion is completely blocked.[28] Confirmation of this model was provided performing FRAP in reconstituted systems at different lipid protein ratios.[15] Another reason why proteins retard long-range diffusion is their immobilizing effect on the surrounding phospholipids.[29,30]

1.2.2 Diffusion-Limited Reactions

In the cellular organization of living organisms the energy of thermal fluctuation, kT, is large enough to perturb the cell's motion.[31] Adam and Delbrück[32] proposed that organisms resolve some of the

problems of timing and efficiency of diffusion of certain molecules by reducing the dimensionality in which diffusion takes place. Membrane-bound diffusion may well compete with transport inside soluble compartments of the cell, which is expected not to be so effective due to the high viscosity of the cytoplasmic matrix. Similar considerations may well be applied to the mitochondrial matrix.

A major aspect of the physiological relevance of diffusion is the control of chemical associations in two dimensions in membranes.

All association processes in solution are ultimately limited by the time it takes to bring reactants together by diffusion. Most macromolecular interactions also require that the molecules attain a correct mutual orientation so that potentially reactive groups are properly aligned; usually the molecules have to collide many times before the reaction takes place.

If the rate of a chemical reaction is limited by the time it takes to bring the reactive groups together *via* diffusion, the reaction is said to be *diffusion-controlled*.[33] Diffusion-limited reactions are viscosity-dependent and have weak temperature coefficients.

According to Smoluchowski,[34] the bimolecular association rate constant, k_a, for two spherical molecules *A* and *B* is

$$k_a = 4\pi N(D_A + D_B) \cdot (R_A + R_B) \tag{1.6}$$

where *N* is Avogadro's number per millimole, D_A and D_B are the diffusion coefficients, and R_A and R_B are the interaction radii of the two molecules.

Since macromolecules are not reactive over their entire surfaces, but on restricted active sites, a full description of the diffusion-limited association process must consider the molecules that are needed for the reaction to occur.[35] It can be assumed that a molecule, owing to the erratic nature of the diffusional path (*random walk*), will come close to its starting point a number of times prior to achieving an appreciable separation from its origin (*microcollisions* as distinguished from *macrocollisions*.[33] Molecules having to come together by diffusion will experience a large number of microcollisions with changes in orientation, facilitating the occurrence of the useful collision.

Feeding the Stokes Einstein relation (Eq. 1.1) in the Smoluchowski relation, if $R_A \approx R_B$, we can approximate

$$k_a = 8kT/3\eta \tag{1.7}$$

which corresponds to $k_a \approx 10^9 - 10^{10}\ \mathrm{M^{-1}s^{-1}}$ under normal aqueous solution conditions. The association could be faster if one molecule is small and diffuses rapidly while the other is large and provides a large target.

If the association reaction depends on a chemical step, the rate constant will depend on both the collision frequency and the rate constant k_c of the chemical step according to the Noyes relation[36]:

$$1/k_a = 1/[4\pi(D_A + D_B)\,(R_A + R_B)] + 1/k_c \tag{1.8}$$

For two spherical molecules, assuming one molecule to be completely reactive, and the other having a reactive patch over its surface, limited by an angle δ_A with the center of the molecule, the diffusion-limited association rate constant will be roughly proportional to sin δ_A:

$$k_A \approx (D_A + D_B)(R_A + R_B)\ \sin(\delta_A/2) \tag{1.9}$$

If the steric constraints are severe (i.e., δ_A is very small) there can be a difference of orders of magnitude with respect to the simple Smoluchowski relation. In real macromolecular associations, however, it is likely that long-range and short-range interaction forces will facilitate and prolong

the collisions, giving the molecules ample opportunity to seek out orientations for reaction. Thus electrostatic and hydrophobic interactions are sources of useful interactions.[37]

It was proposed that reduction of dimensionality from three to two dimensions, as usually happens in membrane-mediated reactions, enhances the rate constants by facilitating collisional encounters.[31] There is some empirical evidence that guided diffusion by reduced dimensionality may be favorable,[38] but the rate constants for two-dimensional diffusion have yet to be rigorously defined. Blackwell et al.[39] developed a two-dimensional analogue of the Stern-Volmer relation for diffusion-limited fluorescence quenching, from which the rate constant could be related to diffusion by the relation

$$k_a = 8Nh(D_A + D_B)\tau f_i/\pi \tag{1.10}$$

where h is the membrane thickness, τ is the lifetime of the excited state of the fluorescent molecule in the absence of the quencher molecule, and f_i is a fitting parameter.

The lateral translational rate of a diffusing molecule is calculated for a bidimensional path by the Einstein-Smoluchowski relation

$$d^2 = 4Dt \tag{1.11}$$

where d is the distance traveled by the diffusing molecule and t is time. However, the mean time τ required to reach a small target of radius R in two dimensions over a distance d, being $d \gg R$, is

$$\tau = (d^2/2D) \ln(d/R) \tag{1.12}$$

The diffusional search for a small target is much more efficient in two dimensions than in three, assuming t to be of comparable magnitude.

1.2.3 Diffusion Control of Enzymatic Reactions

In the Briggs-Haldane steady-state approximation in a monosubstrate enzyme reaction, the initial velocity v_o is expressed by

$$v_o = k_3[E_t][S]/[(k_2 + k_3)/k_1] + [S] \tag{1.13}$$

where E_t is the total enzyme concentration, S is substrate, and k_2 and k_3 are first-order rate constants, whereas k_1 is the second-order rate constant of enzyme-substrate reaction; $(k_2 + k_3)/k_1$ is the *Michaelis-Menten constant, K_m*, and k_3 is equated with the *catalytic constant* k_{cat}. Equation 1.13 can therefore be rewritten in the classical form of the Michaelis-Menten equation:

$$v_o = k_3[E_t][S]/K_m + (S) \tag{1.14}$$

The ratio k_{cat}/K_m is a very useful parameter in enzyme kinetics, because it represents the *minimal* value of the bimolecular reaction rate constant of enzyme with substrate, k_{min}, approaching the true k_1:

$$k_{min} = k_{cat}/K_m = k_1 \bullet k_3/k_2 + k_3 \tag{1.15}$$

The binding of substrates by many enzymes is fast, and it is possible that it proceeds at rates limited by the diffusion of the reactants: high values of k_1 (and hence of the k_{min} value, which is easily accessible in steady-state kinetic analysis) are suggestive of a diffusion-limited enzyme reaction.[40]

Diffusion-controlled enzymatic reactions have low activation energies. Moreover, Equation 1.7 shows that diffusion-limited reactions are predicted to be sensitive to medium viscosity.

For a diffusion-limited associaton rate constant of an enzymatic reaction we have

$$k_{\min} = f(4\pi RATN)/\eta \tag{1.16}$$

where A is an empirical constant and f is a steric factor expressing the efficiency of useful collisions to determine the reaction with respect to total collisions. The percent collisional efficiency for enzymatic reactions is usually low (1% or less).

The variation of the kinetic parameters of an enzyme with solution viscosity can be used to evaluate the extent to which the magnitude of K_m is determined by the rate constant for diffusion-controlled encounters between substrate and active site.

The activation energy for aqueous diffusion is low, and therefore diffusion-limited reactions usually exhibit low activation energies. It should be noted that an Arrhenius plot of log $k_{\min}$ vs. $1/T$ should be curved if the diffusion-limited component and k_c (see Eq. 1.8) have different activation energies. Nonlinear Arrhenius plots have been found in soluble enzymes, and more often in membrane-bound enzymes,[41] but they were usually interpreted in terms of transition temperature of the lipids or of conformational changes of the enzyme.

In terms of enzyme kinetics, the effects of temperature and viscosity on k_1 in a diffusion-limited reaction become experimentally apparent in the K_m (being $K_m = k_2 + k_3/k_1$) and not usually in the k_{cat} (being $k_{cat} = k_3$ mainly indicative of the endogenous chemical activation step in catalysis). Nevertheless, insofar as k_3 contains the products dissociation step(s), diffusion control may exert changes in k_{cat} if product release from the active site is diffusion-limited.

In terms of a microscopic reaction scheme, diffusion control means that the substrate molecules are used by the enzyme at a rate faster than they can be replenished by diffusion from the bulk solution. Thus the bulk concentrations of substrate necessary to progressively saturate the enzyme are increased and the apparent K_m, therefore, increases. On the other hand, at infinite substrate concentration, the local substrate concentration is also infinite and therefore V_m does not change.

Not all reactions where k_{cat}/K_m change with viscosogens need necessarily be diffusion-limited, and the use of *poor substrates*[42] is an important control to ensure that observed rate effects by viscosogens are really due to viscosity changes. If a good substrate reacts near the diffusion limit and the reaction is sensitive to solution viscosity, a poor substrate for which the rate of reaction is determined by a slow chemical step should be insensitive to viscosity.

1.3 ORGANIZATION OF THE MITOCHONDRIAL INNER MEMBRANE

The isolation of discrete lipoprotein redox complexes from the inner mitochondrial membrane and the finding that the respiratory chain could be reconstituted from the isolated complexes led Green[43] to postulate that overall respiratory activity is the result of both intracomplex electron transfer in solid state between redox components having fixed static relations and, in addition, of intercomplex electron transfer ensured by rapid diffusion of mobile components acting as cosubstrates, i.e., Q and cytochrome c (cyt. c). This view was substantially confirmed over the following years,[44] although the organization of the respiratory chain was enriched with an increasing number of respiratory complexes[45] (cf. Figure 1.1).

1.3.1 Models of Structural Organization

Two extreme conditions can be envisioned for the organization of the respiratory chain.[46] In the first view, the chain is organized in a *liquid state*. The large enzymatic complexes are randomly distributed in the plane of the membrane, where they move freely by lateral diffusion. Ubiquinone

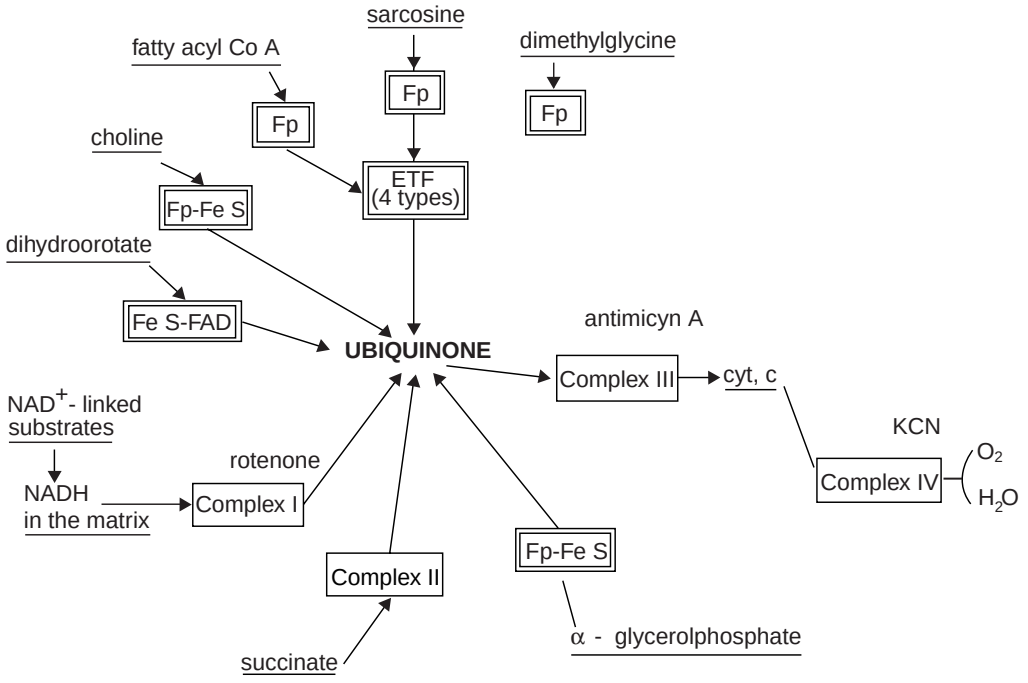

FIGURE 1.1 Schematic representation of the respiratory chain of bovine heart mitochondria showing the central role of Coenzyme Q.

and cyt. c are also mobile electron carriers, whose diffusion rate is faster than that of the bulkier protein complexes; their diffusion-coupled collision frequencies may be either higher or lower than any given reaction step within the complexes, and consequently electron transfer would be either reaction-limited or diffusion-limited. Alternatively, the components of the chain are present as aggregates, ranging from small clusters of few complexes to the extreme of a *solid-state* assembly. The aggregates may be either permanent or transient, but their duration in time must be larger than any electron transfer turnover in order to show kinetic differences from the previous model.

The *random collision model* has been systematically elaborated on by Hackenbrock,[47] who provided convincing evidence that respiratory complexes undergo independent lateral diffusion, and electron transfer is a diffusion-coupled kinetic process. The same group postulated that electron transfer is limited by diffusion of the faster components (Q and cyt. c).[15]

The view of a solid-state arrangement is scarcely tenable. However, the possibility of transient aggregates[48] and of preferential associations between complexes (e.g., 49) deserves some consideration.

The lateral diffusion of protein complexes in mitochondrial membranes was first measured[50] by a combination of postfield relaxation and freeze-fracture electron microscopy, yielding D_l of $8.3 \cdot 10^{-10}$ cm^2/s for the particles in spherical mitoplasts. Later, Gupte et al.[51] reported values near $4 \cdot 10^{-10}$ by FRAP on labelled respiratory complexes in megamitochondria, whereas Hochman et al.[48] also using FRAP, obtained D_l of $1.5 \cdot 10^{-10}$ cm^2/s for cytochrome oxidase in megamitoplasts from cuprizone-fed mice.

The diffusion of integral membrane proteins protruding into aqueous compartments is affected by the viscosity of the aqueous matrices,[52] in accordance with the Saffman-Delbrück relation. On the other hand, the long-range diffusion measured by FRAP (μm) could be slower than short-range diffusion (nm) as a result of the high density of proteins. Accordingly, the D_l of Complex III was strongly enhanced by phospholipid enrichment of the membrane;[15] significantly, the long-range D_l of phospholipids, though also increased, was affected to a much smaller extent.

1.3.2 Diffusion of Ubiquinone

Direct evidence for the localization of Q homologs was derived from studies in oriented bilayers by a linear dichroism technique.[53,54] The presence of two partly overlapping opposite-signed bands suggested two main orientations of the polar head of the quinone molecules, one situated in the hydrophobic interior and the other near the membrane surface. The available data supported a model where most of the Q molecules are located in the membrane midplane, with the headgroup oscillating transversally across the membrane. The transversal movement of the Q molecule would most likely be limited to oscillations of its relatively polar headgroup, allowing interactions with water-soluble redox reagents.[55,56] These interactions, however, appear to be relatively inefficient.[57]

We attempted to confirm the extended conformation of the Q molecules by performing a molecular dynamics simulation in the vacuum starting from different initial configurations. In all cases, the simulation yielded a folded structure for Q_{10} and other long isoprenoid chain homologs.[58,59] A significant energy difference was obtained between the folded and extended structure of Q_{10}, indicating a much higher stability of the folded conformation (F. Andriani, R. Fato, and G. Lenaz, unpublished) (Figure 1.2). The size of folded Q_{10} is surprisingly similar to that of short chain quinones in an extended configuration, with a length of approximately 21 Å. The cutoff for folding of the

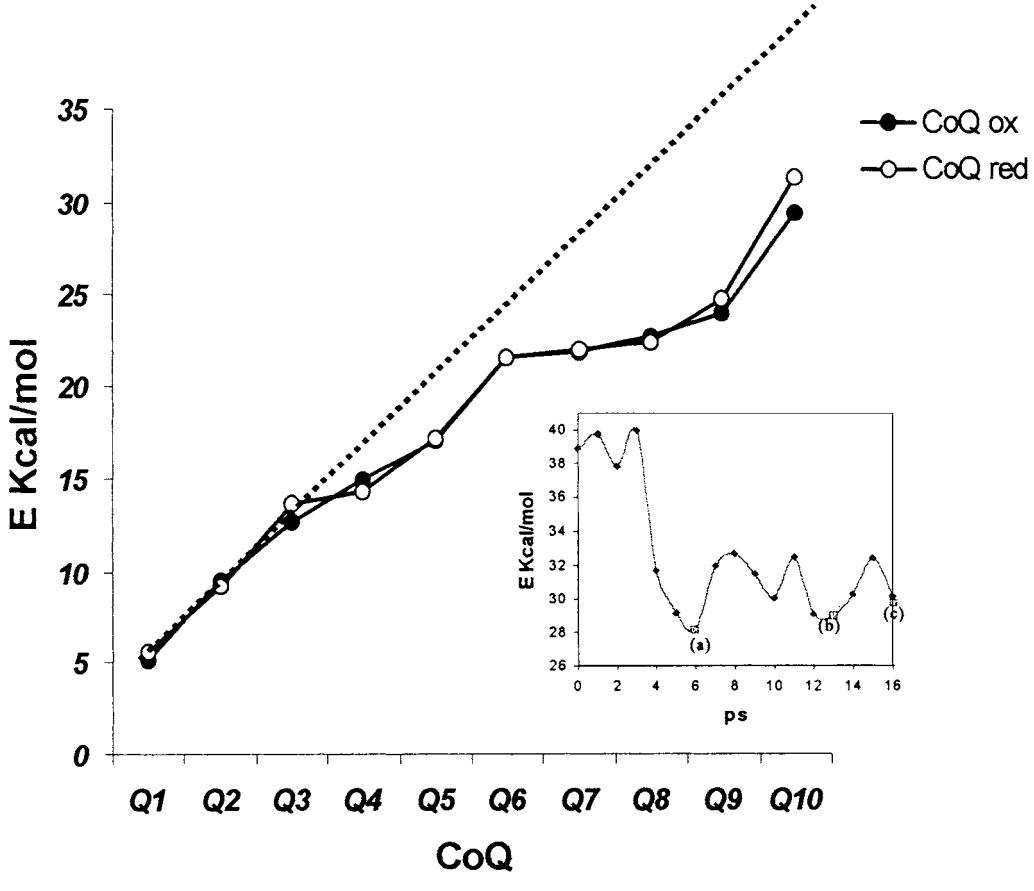

FIGURE 1.2 Energetic comparison (Kcal/mol) of the series CoQ_{1-10} in the oxidized and reduced forms. The dotted line represents energy levels of the unfolded conformations, proportional to the numbers of isoprenoid units. The inset exhibits a molecular dynamics simulation at constant temperature showing the three structures having the lowest energy levels.

TABLE 1.1
Experimental and Calculated Diffusion Coefficients of Some CoQ Homologs

CoQ Homologs	V(A³) (Folded)	Theoretical $D_l(10^{-7}cm^2/s)$		Experimental $D_l(10^{-7}cm^2/s)_a$
		(Folded)	(Linear)	
Q_1	191.0	_	50	33.0
Q_6	595.8	4.47	20.5	8.5
Q_9	676.3	1.29	5.3	3.2
Q_{10}	531.7	7.98	0.9	3.9

[a]Experimental D_l were obtained by fluorescence quenching of a pyrene-phospholipid derivative (cf. Di Bernardo et al., 1998).

isoprenoid chain is at 4 isoprenoid units; the bond energy of the ubiquinone molecule as a function of isoprenoid chain length gives a plateau at the same number of 4 isoprenoid units. No significant difference in these parameters was found for oxidized and reduced ubiquinones. These results are in agreement with EPR and ENDOR studies on Q semiquinones.[60] On the other hand, in a recent study combining different biophysical techniques, Gomez-Fernandez et al.[61] suggested that the location of Q in the center of the bilayer might be forced by formation of head-to-head aggregates. However, a similar result could be achieved by a folded conformation.

There are important implications of a folded structure. First, protein binding during electron transfer may require unfolding, contributing to a high activation energy and low collision efficiency of electron transfer. Moreover, the similar sizes of short and long homologs would explain the similarity of diffusion coefficients found in our laboratory for all quinone homologs[58,62] (Table 1.1).

Gupte et al.[51] measured the D_l of a fluorescent derivative of a decyl-Q analog by FRAP, reporting a value of $3 \cdot 10^{-9}$ cm²/s; the same coefficient was found[63] using a fluorescent derivative of the natural homolog Q_{10}. On the other hand, in protein-free lipid vesicles, the D_l of the short derivative used by Gupte et al. was $3 \cdot 10^{-8}$ cm²/s,[64,65] one order of magnitude faster, in accordance with the lack of the obstructing effect of proteins in the diffusion path.[15]

Exploiting collisional fluorescence quenching of membrane-bound fluorophores by oxidized ubiquinone homologs, Fato et al.[62] calculated $D_l > 10^{-6}$ cm²/s in both liposomes and mitochondrial membranes, using calculations to account for the partition and effective concentration of the quencher in the membrane and using the Smoluchowski relation [Equation 1.6] for calculating D_l from the second order rate (quenching) constants. Subsequently, using Eq. 1.7, Blackwell et al.[39] calculated $D_l > 10^{-7}$ cm²/s for plastoquinone in lipid vesicles. Using the latter relation, Lenaz et al.[66] recalculated D_l of $4 \cdot 10^{-7}$ cm²/s on their previous experiments, and found additional evidence for values in that range.[67]

By exploiting an electrochemical technique in artificial lipid bilayers, Marchal et al.[68] measured D_l of $2 \cdot 10^{-8}$ cm²/s, two to three times smaller than the corresponding values for lipids in the same bilayer.[69] This unexpected finding points out that the bilayer used may not represent a reliable model of a natural membrane.

The obstructing effect of proteins on diffusion was found by Blackwell and Whitmarsh[70] also by the quenching technique, however, it was not confirmed by Di Bernardo et al.[58] either in phospholipid-reconstituted cytochrome oxidase or in bovine submitochondrial particles.

The differences between D_l measured by FRAP and fluorescence quenching are very large and can be only partly ascribed to their different range of measurement. In mitochondrial membranes, the high protein density may affect long-range diffusion measured by FRAP more than short-range diffusion measured by fluorescence quenching.[21] Quinone binding to proteins might be responsible

for retarding diffusion, but this retardation would increase proportionally to the length of the diffusion path measured.

In lipid vesicles there is still one order of magnitude difference between FRAP and fluorescence quenching in measuring Q diffusion. The explanation of this discrepancy may be in the different range explored by the FRAP and quenching techniques, or in the fact that FRAP has been performed using modified quinones that are presumably located on the membrane surface, where the effect of obstacles may be much more pronounced.[59] In fact, the FRAP technique requires the use of fluorescent derivatives of the quinones, which were found to have a nonfolded conformation in our molecular simulation. Such a conformation is compatible with only very slow diffusion rates according to the free volume theory. Moreover, such a structure is likely to move on the membrane surface, where the rate of void formation is much lower than in the midplane and the viscosity is therefore very high.

Ubiquinone diffusion measured by fluorescence quenching is not affected by the viscosity of the outer medium[62] in accordance with its location in the hydrophobic core of the membrane (e.g., 53) and then not subjected to drag from the outer medium.[71] Hackenbrock et al.[15] reported that high sucrose retarded Q diffusion measured by FRAP. This effect, however, may be secondary to membrane physical rearrangements due to the dehydrating effect of poly-hydroxy-alcohols[72,73] decreasing the availability of voids for the migration of the quinone in the lipid bilayer.[74]

1.4 DIFFUSION COUPLING OF UBIQUINONE

The first proposal that Q functions as a mobile electron carrier was made by Green[43] on the basis of the isolation of discrete lipoprotein complexes of the respiratory chain, of which the quinone was a substrate in excess concentration over the prosthetic groups in the complexes, and was subsequently supported by the kinetic analysis of Kröger and Klingenberg.[75] They showed that steady-state respiration in submitochondrial particles from beef heart could be modeled as a simple two-enzyme system, the first causing reduction of ubiquinone and the second causing oxidation of ubiquinol. If diffusion of the quinone and quinol species is much faster than the chemical reactions of Q reduction and oxidation, the quinone behaves kinetically as a homogeneous pool. According to this assumption, during steady-state electron transfer, the overall flux observed (V_{obs}) will be determined by the redox state of the quinone and described by the *pool equation*

$$V_{\text{obs}} = (V_{\text{red}} \bullet V_{\text{ox}})/(V_{\text{red}} + V_{\text{ox}}) \tag{1.17}$$

This expression was verified under a wide variety of input and output rates and establishes that Q distributes electrons randomly among the dehydrogenases and the bc_1 complexes, behaving indeed as a freely diffusable intermediate. The hyperbolic relation of electron flux on the rate of either Q reduction (V_{red}) or QH_2 oxidation (V_{ox}) was confirmed in a variety of systems.[76,77]

1.4.1 Ubiquinone Saturation Kinetics

If the Q concentration is not saturating for the activity of the reducing and oxidizing enzymes, the equation is modified[77] by feeding it in the Michaelis-Menten equation for enzyme kinetics, taking into account Q_t concentration, the individual V_{max} of the dehydrogenase and bc_1, and their dissociation constants for Q. V_{obs} is hyperbolically related to $[Q_t]$ and maximal turnovers of electron transfer are attained only at $[Q_t]$ saturating both V_{red} and V_{ox}.[21]

Direct titrations of Q-depleted mitochondria reconstituted with different Q supplements yielded a "K_m" of NADH oxidation for Q_t in the range of 2–5 nmol/mg mitochondrial protein,[78] corresponding to a Q_t concentration of 4 to 10 mM in the lipid bilayer. The "K_m" in the composite system is a poised function of V_{max} and dissociation constants for Q of the complexes involved. This "K_m" can therefore vary with rate changes of the complexes linked by the Q-pool, but is

nevertheless an important parameter, in that it is operationally described as the Q_t concentration yielding half-maximal velocity of integrated electron transfer V_{obs}.[79] Analysis of the literature shows that the physiological Q content of several types of mitochondria[80] is in the range of the K_m for NADH oxidation, and therefore not saturating for this activity.

In contrast to NADH oxidation, the "K_m" for succinate oxidation for Q_t was found one order of magnitude lower,[78] although Norling et al.[81] had found similar values for the two systems.

The relation between electron transfer rate and Q concentration was seen in reconstituted systems and in phospholipid-enriched mitochondria for NADH oxidation.[82,83] Although NADH oxidative activities higher than the physiological rates could be attained by enriching the membranes with extra ubiquinone, the theoretical $V_{obs(max)}$ cannot be reached experimentally. The reason could be in the limited miscibility of ubiquinone with phospholipid bilayers. Two-phase systems are formed just above the physiological Q concentration;[57,84,85] clustered ubiquinone would be kinetically inactive, and clustering would impose an upper limit on the electron transfer rate in the Q region.

Q-pool behavior does not exclude the existence of an aliquot of quinone molecules that are not freely diffusable but are tightly bound to the complexes. Evidence exists for bound quinones[86] and for Q-binding proteins within the complexes.[87] Bound ubiquinone participates in intracomplex electron transfer as a prosthetic group of the enzymes, and actually much evidence was obtained in favor of semiquinone forms stabilized by protein binding. This bound quinone is a prerequisite of the mechanisms of electron flow in the bc_1 complex[88] and in Complex I.[89]

1.4.2 Determination of the Kinetic Constants of Enzymes Using Hydrophobic Substrates

The investigation of diffusion control is mainly based on the evaluation under different conditions of the k_{cat}/K_m ratio, in which a concentration unit is present.

Several membrane-bound enzymes utilize hydrophobic substrates reacting with the active sites from within the lipid bilayer. Their kinetics are complicated by incomplete knowledge of the true substrate concentration in the membrane. In some cases, partly water-soluble substrates are used to overcome the difficulties in handling the hydrophobic natural substrates. These homologs partition from the aqueous medium into the lipid phase, where their concentration is a function of their partition coefficient and of the relative fraction of the membrane volumes in the total assay medium.

Kinetic methods to calculate the true Michaelis constants of hydrophobic substrates and the partition coefficients in the membrane or in detergent micelles have been implemented by performing saturation kinetics experiments at different membrane fractional volumes.[90,91,92] Using this approach, Fato et al.[90] obtained the following relation:

$$K_{app} = \alpha(K'_m - K'_m/P) + K'_m/P \tag{1.18}$$

where K_{app} is the experimental *apparent* K_m, which on its hand is related to the *true* absolute K_m, K'_m, to the partition coefficient P and to the relative volume α (volume lipids: volume water).

A plot of K_{app} vs. α allows the simultaneous determination of both the partition coefficient P and the true K_m of the enzyme. The K_m, as well as the substrate concentration, may be expressed in mol • l^{-1} of membrane lipids (or, alternatively, as the mole fraction of substrate in the membrane lipids).

If the substrate molecules interact with the active site of the enzyme from within the bilayer, addition of extra lipids to the assay medium increases the value of α by increasing the lipid phase and decreases the substrate *concentration* (although increasing its total amount) in the lipid phase, resulting in the observation that K_{app} increases with an increase in the concentration of total lipid in the assay medium.

1.5 ROLE OF DIFFUSION IN THE OXIDATIVE PHOSPHORYLATION SYSTEM

In the oxidative phosphorylation machinery, diffusion of substrates and substrate-like molecules should play a role in the following compartments: (a) NAD^+/NADH, most substrates for the dehydrogenases, and ADP/ATP in the matrix; (b) ubiquinone in the lipid core of the inner membrane; (c) cytochrome c and glycerol-3-P in the intermembrane space (with glycerol-3-P dehydrogenase being the only primary dehydrogenase with its active site on the cytoplasmic side).

Both mobile intermediate components of the respiratory chain, ubiquinone and cyt. c, have been considered for diffusion control.[15] They are respectively contained in the inner membrane lipid bilayer and in the intermembrane space, and the sources of possible diffusional constraints are obviously different. In the case of ubiquinone, the viscosity of the membrane[15] and the crowding of the diffusion path by proteins[28] are possible sources of slowing diffusion. As for cyt. c, it is contained in the intermembrane space; being a basic protein, it can engage electrostatic binding with the inner membrane,[93] both with phospholipids and with protein components of Complexes III and IV. The cyt. c interacting with phospholipids diffuses laterally on the membrane surface in two dimensions. At high ionic strength, however, the diffusion changes progressively to pseudolateral and totally three-dimensional.[15] This behavior is obtained in mitoplasts where the bulk ionic strength is changed by KCl. Although it is reasoned that high KCl may be present in the intermembrane space, with it being in contact with the cytoplasm, it is not clear what the behavior can be of this protein in a highly crowded environment. It must be considered that the intermembrane space is a virtual space under most physiological conditions, containing several other soluble proteins besides cyt. c itself, and hosts the peripheral cytoplasmic domains of the intramembrane complexes. The ionic activity in such a space is difficult to evaluate, and doubts may be raised on the likelihood of free tridimensional diffusion for cyt. c. The possibility of small restricted oscillations between the active sites of cyt. c reductase and oxidase should be considered. The relevance of these studies to the physiological situation is still uncertain, at least where the role of diffusion is questionable.

1.5.1 Diffusion Control of Individual Redox Reactions

Although many investigations were made on diffusion-limited steps in the activity of soluble enzymes, few studies are available on possible diffusion-limited steps in membrane enzymes.

In a study of solubilized cytochrome oxidase using media of different viscosities, Hasinoff and Davey[93] found that the interaction of reduced cyt. *c* with the enzyme is partly diffusion-controlled.

The interaction of the membrane-bound bc_1 complex with its substrates, ubiquinol and cytochrome c, was studied by Lenaz.[67,94] The diffusion-limited collisional frequency of ubiquinol with the complex, calculated using the Smoluchowski relation or analogous two-dimensional equations is $>10^8 - 10^9\ M^{-1}s^{-1}$ using as D_l for ubiquinol, the short-range value of 10^{-7} to 10^{-6} cm^2/s. This corresponds to about 10^{18} collisions s^{-1} cm^{-2}. The corresponding values obtained by Hackenbrock et al.,[15] using a diffusion coefficient for ubiquinone of 3×10^{-9} cm^2/s obtained by FRAP, were in the range of 10^{13} to 10^{14} collisions s^{-1} cm^{-2}.

Ubiquinol cyt. *c* reductase activity in mitochondrial membranes is necessarily investigated using short-chain ubiquinol homologs,[95] which dissolve in the membrane by partitioning from the water phase before reaching the active site. Using the method of Fato et al.[90] to calculate the true K_m expressed as substrate concentrations in the membrane, by performing saturation kinetics at different membrane fractional volumes in the medium, the true $k_{min} = k_{cat}/K_m$ were in the range of $1.8 \times 10^4\ M^{-1}s^{-1}$ for ubiquinol-1 and $6.3 \times 10^4\ M^{-1}s^{-1}$ for ubiquinol-2.[94]

The only rate constant available for the natural ubiquinol-10, measured in bacterial chromatophores after flash activation of the reaction center, is $3 \times 10^5\ M^{-1}s^{-1}$.[96] The same value is obtained by calculating k_{cat}/K_m for ubiquinol-10 in beef heart mitochondria using the turnover number of

370 s^{-1} [46] and the K_m for ubiquinol-10 of 0.6 nmol/mg protein,[97] corresponding to ca. 1.2 mM in the lipid phase.

These values of the bimolecular rate constants of enzyme-substrate association are orders of magnitude smaller than the diffusion-controlled collision frequencies (as obtained from the fluorescence quenching experiments); thus, either the process is reaction-controlled or it is diffusion-controlled with very low collision efficiency.

The dependence of the k_{cat}/K_m ratio in membrane-bound ubiquinol cyt. c reductase on the viscosity of the aqueous medium, varied by agents increasing viscosity, and investigated by Noyes, plots according to Eqs. 1.7 and 1.8, showed the presence of negligible diffusion control component for ubiquinol-1 and -2, whereas most of the k_{cat}/K_m ratio for cyt. c appeared to be diffusion-limited.[98] The diffusion-limited component for cyt. c was exhibited at both low and high ionic strength, indicating that both bidimensional and three-dimensional diffusion were rate-limiting.

The K_m increase of the reductase for cyt. c at increasing viscosity was concomitant with an apparent decrease of the K_m for ubiquinol. The increase of K_m for the more limiting substrate, accompanied by a decrease of K_m for the less limiting substrate, was described as typical for an enzyme using two substrates limited by diffusion of one of them.[99] This behavior agrees with the hypothesis that cyt. c diffusion is limiting for ubiquinol cyt. c reductase.

The presence of diffusion limitations for water-soluble substrates in immobilized enzymes was widely investigated. Diffusion limitations in unstirred layers or in the matrix of immobilized supports lead to nonlinear saturation kinetics (e.g., 100), K_m increase for the more limiting substrate accompanied by K_m decrease for the other,[99] and discontinuities in the Arrhenius plots with decreased activation energy at high temperature.[101] All of these properties have been found in ubiquinol cyt. c reductase *in situ* in mitochondrial membranes.[94] In particular, discontinuous Arrhenius plots are a characteristic feature of membrane-bound enzymes.[41,102] Other possible reasons for breaks in Arrhenius plots are changes in the rate-limiting step in the chemical reaction path, temperature-dependent conformational changes, phase changes of the phospholipids, or viscosity becoming rate-limiting for conformational flexibility.[103] The break found in the Arrhenius plot of the *apparent* V_{max} of ubiquinol cyt. c reductase in the membrane state (but not in the isolated form) was discovered to have originated from the steep increase of the K_m for cyt. c with temperature, resulting from diffusion control.[98]

The Arrhenius plots of the k_{min} (k_{cat}/K_m) of ubiquinol cytochrome c reductase either *in situ* or embedded in liposomes were linear for both ubiquinol-2 and cyt. c, with activation energies of 5.7 and 1.3 kcal/mol, respectively. The activation energy of $k_{min(ubiquinol)}$ was even higher (14 kcal/mol) when evaluated for ubiquinol concentration in the lipid phase.[94] The activation energy of $k_{min(cyt.c)}$ closely agrees with that of aqueous diffusion of water-soluble molecules, confirming that cyt. c diffusion may be rate-limiting. On the other hand, the activation energy of short-range Q diffusion, calculated by fluorescence quenching, was 1 to 2 kcal/mol,[62] in contrast with the much higher value for $k_{min(ubiquinol).}$ Higher values (9 to 12 kcal/mol) were reported by Chazotte et al.[104] for long-range Q diffusion by FRAP, compared with those found by fluorescence quenching. In the case of an individual enzyme, there is no doubt that only short-range diffusion of its substrates is meaningful. It appears from the large difference existing between the activation energy of short-range Q diffusion and that of k_{min} for ubiquinol of ubiquinol cyt. c reductase that the collision frequency is much greater than the observed association rate constant. This argues against the activity of ubiquinol cyt. *c* reductase to be limited by ubiquinol diffusion to the active site. The same conclusion was reached by Crofts[96] studying the activation energy of cyt. *b* reduction by endogenous ubiquinol in ubiquinol cyt. c_2 reductase of *Rps. sphaeroides*.

Cholesterol incorporation in the membrane of submitochondrial particles or in liposomal bc_1 complex, by enhancing bilayer viscosity, lowered D_l for ubiquinones,[62,94] but had no effect on either k_{cat} or $k_{min(ubiquinol)}$. It is unlikely that the enzyme was confined into fluid patches of pure phospholipids separated from cholesterol. In fact, the cholesterol level incorporated in the bc_1 proteoliposomes

TABLE 1.2
Evidence Concerning Diffusion Control Steps in Ubiquinol Cyt. *c* Reductase *in Situ* in Bovine Heart Mitochondrial Particles

1. Ubiquinol (using ubiquinol-2)
a) $k_{cat}/K_m \approx 2–5 \times 10^8\ M^{-1}s^{-1}$ calculated in assay medium but only $\approx 1–3 \times 10^4\ M^{-1}s^{-1}$ calculated in the lipid phase compared with collisional frequency of $10^9\ M^{-1}s^{-1}$ in the lipid phase
b) High temperature dependence of k_{cat}/K_m ($\approx$14(Kcal/mol) calculated in the lipid phase)
c) Little dependence on viscosity of the assay medium
d) No dependence on membrane viscosity as changed by cholesterol (compared with lowered D_l)

2. Cytochrome *c*
a) $k_{cat}/K_m\ 8 \times 10^7\ M^{-1}s^{-1}$
b) Increased K_m from soluble enzyme to membrane-bound enzyme
c) Low temperature dependence of k_{cat}/K_m (1–2 Kcal/mol)
d) High dependence on viscosity of the assay medium

(1:2 molar ratio with phospholipids) should allow a uniform distribution of the sterol in the bilayer,[105] although Chazotte et al.[104] found evidence of lateral phase separations in dimyristoyl lecithin/cholesterol bilayers. Alternatively, however, the mitochondrial membrane fluidity could be increased in *S. cerevisiae* by changes in the fatty acid unsaturation index obtained by growing the cells at low temperature, with the expected increase of Q diffusion.[67] Under such conditions, however, no changes in $k_{min(ubiquinol)}$ could be observed.

The conclusion was reached that ubiquinol cyt. *c* reductase has a diffusion-limited component for aqueous diffusion of the acceptor substrate, cyt. *c*, but is not limited by membrane diffusion of the donor substrate, ubiquinol (Table 1.2).

A subsequent study on bovine heart Complex I (NADH CoQ reductase)[106] demonstrated that the k_{min} for CoQ_1 is 4 orders of magnitude lower than the bimolecular collision constant calculated from fluorescence quenching of membrane probes. Moreover, the activation energy calculated from Arrhenius plots of k_{min} is much higher than that of the collisional quenching constants. These observations strongly suggest that the interaction of exogenous quinones with the enzyme is not diffusion controlled; likewise, as analyzed for Complex III (see above), the interaction with endogenous CoQ_{10} is also not limited by its diffusion in the membrane.

1.5.2 Diffusion Control of Integrated Electron Transfer

It may be reasoned that ubiquinone diffusion, though not rate-limiting for the individual Q-reactive enzymes, becomes rate-limiting in the integrated function of the Q pool, where the overall combined activity of two enzymes is constrained by the new parameter of intercomplex separation directing the reduced Q molecules toward Complex III and the oxidized ones back to Complex I or II. The diffusion path is run in a time proportional to the square of the intercomplex average distance.[31] Whereas in the individual enzymic activities short-range diffusion (<10 nm) is involved, in integrated electron transfer, the diffusion process takes place on a average over a path of several nanometers. From the concentrations of electron transfer complexes in the mitochondrial membrane, average distances can be calculated through which the randomly distributed redox components must diffuse to effect a consecutive reduction and oxidation (Lenaz, 46) (Table 1.3).

A rough calculation indicates that in an area of 900 nm^2, scanned by a reduced Q molecule leaving Complex I to reach Complex III, assuming a distance of 30 nm, there are about 12 protein molecules and 800 lipid molecules, if lipids occupy 60% of the total area. According to Eisinger et al.[28] the long-range diffusion coefficients are slowed in proportion to the area covered by obstacles and to the reciprocal of their size. For a relative protein area of 40%, with obstacles equated with hexagons having sides of length three times the lipid–lipid separation (equivalent to proteins having

TABLE 1.3
Average Distances Between Respiratory Complexes in Bovine Heart Mitochondria (cf. Lenaz 1988)

Redox Complexes	Total Concentration of Redox Complexes[a] (molecules/cm² membrane) × 10^{-10}	Minimum Distance between Complexes (nm)[b]
Complex I	2.6–5.2	19–27
Complex III	10.8–22.8	
Complex II	8.2	18–22
Complex III	10.8–22.8	
Complex III	10.8–22.8	12–16
Complex IV	25.8–43.9	

[a] Calculated from the contents given by Capaldi (1982), assuming phospholipids with an average molecular weight of 750 Kda to occupy an area of 70 A²/molecule (corresponding to 70 A²/two molecules in a lipid bilayer).
[b] Calculated by the formula $(c_1 + c_2)^{-1/2}$, where c_1 and c_2 are the concentrations of the two partner complexes in molecules/cm² membrane.

a radius of 2.4 nm), the long-range diffusion coefficient of a molecule dissolved in the lipid phase would be lowered to 1/3 of the unobstructed value.

In accordance with the above theory, Hackenbrock et al.[15] reported that lipid diffusion is enhanced about fourfold by a sevenfold phospholipid enrichment of mitochondrial membranes. On the contrary, Schindler et al.,[107] also using FRAP in *E. coli* reconstituted membranes, found that on a range of protein concentration of 0–60% by weight, D_l for phospholipids remained essentially constant, whereas D_l for lipopolysaccharide decreased over tenfold. Although the concentration and hence the cross-sectional area of integral proteins must be essentially similar in mitochondrial membranes and in *E. coli* reconstituted membranes, it appears from the two studies that phospholipid mobility was more severely affected by protein concentration in the study by Hackenbrock et al.

A possible explanation may lie in a stronger interference of the indocarbocyanine dye used by Hackenbrock et al.[15] with the peripheral portions of the proteins in comparison with the nitrobenzoxadiazole derivative used by Schindler et al.,[107] in line with a lower D_l of the former[108] and with the Saffman-Delbrück dependence on viscosity of the outer medium. The same hypothesis would explain why diffusion of lipopolysaccharide, having a wide extramembrane moiety, is dramatically inhibited by increased protein concentration.

The uncertainties of the significance of protein crowding on obstruction of the diffusion path for small hydrophobic molecules like ubiquinone do not allow us to predict from either short-range and long-range diffusion coefficients, whether electron transfer in the inner mitochondrial membrane is diffusion controlled.

Hackenbrock[15,109] approached the problem kinetically by comparing the temperature dependence of the overall steps (diffusion plus chemical reaction) in the Complex II-ubiquinone-Complex III span in the uncoupled inner membrane. The activation energy for the overall diffusion steps for the II-Q-III span was calculated to be 12.2 kcal/mol, as compared with E_a of 12.9 kcal/mol for succinate cyt. c reductase activity. The finding was interpreted as compatible with diffusion control of this electron transfer span. Furthermore, when the protein–lipid ratio was decreased by phospholipid enrichment, the activation energies of both lateral diffusion and electron transfer decreased in proportion to the degree of enrichment. The uncertainties concerning the role of the Q pool in succinate oxidation and the effect of the Q substrate dilution on activation energy of the enzyme *rate* (not the substrate-enzyme association rate constant) make these conclusions doubtful. Similarly, Hackenbrock concluded that the rate-limiting step of duroquinol oxidase activity is the diffusion

step of cyt. c to cytochrome oxidase. The increase of the rate of electron transfer catalyzed by cyt. c by increased ionic strength was taken to mean that the diffusion rate-limiting step of cyt. c is relieved by shifting from two- to three-dimensional diffusion.

The interpretation of studies concerned with activation energies alone in complex systems is subject to considerable uncertainty, considering that most mitochondrial enzymes that do not use ubiquinone have a similar range of E_a.[41] The activation energies of integrated electron transfer activities using the Q pool are usually within a range close to the E_a of an individual enzyme as ubiquinol cyt. c reductase.[46]

Although a diffusion-limited component may appear in the activity of individual enzymes working at very high turnover numbers (cf. previous section for cyt. c), when electron transfer is integrated through a common substrate pool, the overall turnover is strongly decreased, reflecting the turnover of the slower enzyme, according to the pool equation[75] and compensating for the possible effect that the obligated distance between complexes using a common intermediate (vs. ubiquinone) imposes on the overall activity. Thus, the integrated reaction would not be diffusion controlled.

The time for a particle to diffuse to a small target in two dimensions is related to the logarithm of the ratio between distance and diameter of the target, according to Eq. 1.12. For a distance of 30 nm, taking D_l of ubiquinone = 4×10^{-7} cm^2/s at room temperature and assuming a diameter of the active site of Complex III of 1 nm, the time for a Q molecule reduced by Complex I to reach Complex III would be 30 μs; for a turnover of 50 s^{-1} (i.e., 20 ms per turnover), close to the physiological rate of NADH cyt. c reductase at room temperature, this time corresponds to over 600 collisions *with the active site* per turnover. The theoretical calculations show that the diffusion limit could be reached only at high turnovers or the chain or over largely increased distances. Using D_l of 5×10^{-9} cm^2/*s* obtained by FRAP, there is still an excess of 7 collisions per turnover.

Assuming a random distribution of the complexes in the lipid bilayer, the increase of the phospholipid content with respect to protein is equivalent to increasing the average distance between complexes. Using this approach for Complexes I and III in reconstituted liposomes, Parenti Castelli et al.[110] found that NADH cytochrome *c* reductase activity is not decreased by increasing intercomplex distance up to 108 nm, at an experimental maximal turnover of 50 s^{-1}. Using Eq. 1.12, this turnover corresponds to a lower limit of D_l of 1.1×10^{-8} cm^2/s in a diffusion-limited reaction having a collision efficiency of 100%. Also the experiments in phospholipid-enriched mitochondria[82] confirm that phospholipid enrichment has no effect on electron transfer provided that the Q concentration is maintained constant by enriching the membranes with liposomes containing ubiquinone as well.

In a further study[59] the distance between complexes was increased by dilution with increasing amounts of phospholipids. A crude mitochondrial fraction containing Complexes I and III was fused by detergent dilution with phospholipids containing different CoQ_{10} concentrations. The increased distance was checked by freeze-fracture electron microscopy, showing that the intramembrane particles were indeed randomly dispersed in the membrane. The experimental distances between intramembrane particles and those expected by calculation from the concentration of the complexes and of the phospholipids were found to closely agree.

The NADH cyt. c reductase activity was affected only at phospholipid contents corresponding to distances over 100 nm between Complex I and Complex III. Theoretical calculation of the collisional frequencies with Complex III of CoQ reduced by Complex I by the Berg relation at different D_l and comparison with the experimental turnovers excluded the possibility of diffusion coefficients of 10^{-9} cm^2/s, as reported by FRAP studies, even at the collisional efficiency of 100%, which is obviously extremely unlikely. Coefficients of 10^{-7} cm^2/s as those we reported are more in line with the experimental turnovers and a low collision efficiency (Figure 1.3).

Using a different approach, Mathai et al.[74] observed that activities depending on the Q-pool are osmotically sensitive, and came to the conclusion that high sucrose prevents formation of voids required for Q diffusion. The result is probably due to the dehydrating effect of sucrose.[72] The lowering by sucrose of diffusion of a fluorescent labeled fatty acid probe was taken as a model behavior for Q diffusion. Since the analogy of diffusion behavior of lipids and Q is questionable,[59] the assumption that electron transfer is rate-limited by Q diffusion is also not proven.

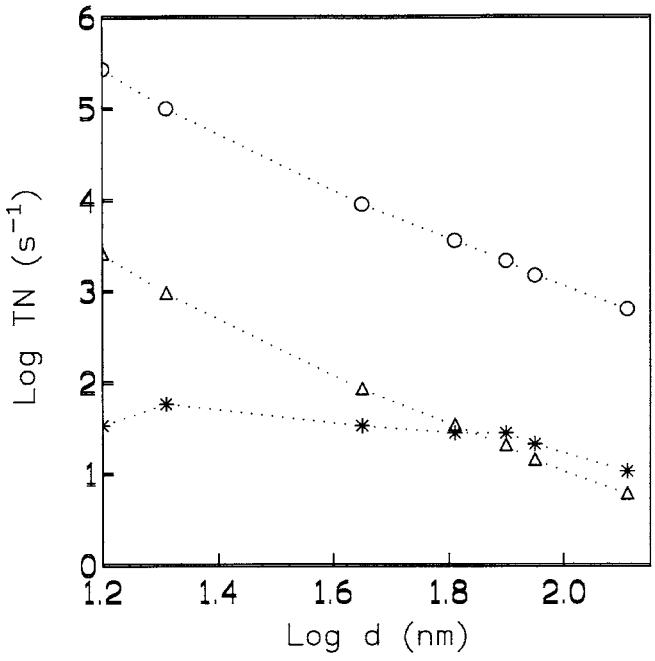

FIGURE 1.3 Comparison between the experimental turnovers obtained for NADH cyt. c reductase activity and the theoretical calculation of the collisional frequencies with Complex III of CoQ reduced by Complex I, calculated according to Berg and Purcell (31) at different D_l and assuming a collisional efficiency of 100%. (*) experimental; (Δ) assuming Dl of 10^{-7} cm^2/s; (O) assuming D_l of 10^{-9} cm^2/s. The data in abscissa are distances between Complex I and Complex III calculated from the contents of the complexes at different phospholipid concentrations.[46]

Another approach to the problem of diffusional control has been to simulate the function of the respiratory chain by establishing a program of random walk of the respiratory carriers in the lipid bilayer. The program, created by G. Palmer, generates a rectangular matrix of 324 points, associating each of them by a random procedure to the respiratory complexes and mobile carriers according to their known sizes and concentrations in the inner membrane of rat liver mitochondria, starting with all components oxidized except Complex I, assumed to be 8% reduced. The program moves the particles according to their bidimensional diffusion coefficients and the relation $s^2 = 4Dt$.

Using a D_l for Q of 4×10^{-7} cm^2/s and a collision efficiency of 0.2% with its redox partners, we obtained a kinetic trace for reduction of the respiratory chain components compatible with available data. In this way we have determined a pseudo-first-order rate constant for CoQ reduction of 180 min^{-1} and a half-time of 231 ms.[59]

We have experimentally confirmed the reduction kinetics of endogenous CoQ by NADH in rat liver submitochondrial particles by presteady-state kinetics using a rapid quenching method.[59] The pseudo-first-order kinetics gives a half-time of about 350 ms, in good accordance with the simulation.

In conclusion, it is clear that membrane fluidity is a prerequisite for diffusion of proteins and other molecules in membranes. However, diffusion-coupled processes do not appear, in general, to be significantly diffusion-controlled.[46] The possible presence of a diffusion-limited step in the interaction of cyt. c with its redox partners, ubiquinol cyt. c reductase and cytochrome oxidase, may not be extended to the situation when the overall respiratory chain is operative. In the intact cell, when the two mitochondrial membranes are tightly apposed, the crowded intermembrane space may forbid cyt. c to freely diffuse, and local pools of cyt. c oscillating between closely packed enzymes may be responsible for electron transfer.

1.5.3 Conclusions on The Mechanisms of Electron Transfer Control

Mobility of the electron transfer components in the mitochondrial respiratory chain represents the main prerequisite for electron flux. A large body of experimental data in isolated mitochondrial membranes demonstrated that ubiquinone and cytochrome c may be used during electron transfer as substrate-like mobile components. In the respiration occurring in intact mitochondria in the functional cell, however, there is no experimental evidence that electron transfer behaves according to completely random collisions. Only double inhibitor titrations[111] have dealt with the problem of mobile intermediates in intact phosphorylating mitochondria. From those studies, doubts were raised as to the "pool" behavior of cyt. c. From studies on the relations existing between Complexes II and III, the possibility of a nonrandom arrangement or even of stoichiometric association can be seriously advanced.

Thus, even if the diffusion coupling principle for electron transfer cannot be generally dismissed, exceptions may be present that strongly complicate the dynamic picture of the respiratory chain.

Although diffusion seems at the least to be an important parameter of respiration, no conclusive evidence is available as to whether it represents a rate-limiting step. Control of respiration exerted by ubiquinone and cyt. c diffusion was proposed by Hackenbrock as one of the postulates of his "random collision model," but the experimental evidence available is probably more against than in favor of diffusion control of respiration.

Among the factors contributing to the rate-limiting step(s) of respiration, the *concentration* of ubiquinone was found to be of importance in NADH oxidation. In fact, ubiquinone concentration is not saturating for NADH oxidation under physiological conditions. This means that any decrease of ubiquinone concentration in mitochondria inevitably induces a decrease in respiratory activity.

The decrease expected in respiratory activity when the ubiquinone concentration in mitochondria is lowered may rationally explain the accumulating literature on clinical efficacy of the quinone exogenously administered in several pathological states (cf. 112).

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2 Topography of Coenzyme Q in Membranes

Peter J. Quinn

CONTENTS

2.1 INTRODUCTION

Coenzyme Q is a fat-soluble component of electron transport chains and, in consequence, it tends to partition into lipophilic organelles of the body such as membranes, lipoproteins, and fat. The coenzyme content of the different organelles varies but the mechanism responsible for regulating the distribution of coenzyme Q is presently unknown. The primary role of coenzyme Q is to transfer electrons between redox components of electron transport chains and thereby to create proton gradients across membranes.[1,2] Additional functions are to act as lipid antioxidants, either directly in its reduced form,[3,4] or to recycle radical forms of vitamin E.[5]

Coenzyme Q is an amphipathic molecule so that in addition to its tendency to partition into lipophilic organelles, it also has a preferred orientation within these structures. Thus the hydroxyl substituents of the benzoquinone ring of ubiquinol prefers a polar environment, whereas the polyisoprene chain achieves lower free energy when located in a hydrophobic environment. This amphiphilic character confers weak detergent-like properties on the molecule, which, in common with other polar lipids such as are found in membranes and lipoproteins, contributes to the overall stability of the structure.

The location and orientation of coenzyme Q in membranes is an important factor in the way it performs its functions. This chapter aims to present evidence on the interaction of coenzyme Q with membranes and, in particular, its distribution within lipid bilayer structures. This will allow conclusions to be drawn as to the effects of coenzyme Q on membrane stability and provide insight into how it performs its different functions.

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2.2 AMPHIPATHIC CHARACTER OF COENZYME Q

The solubility of coenzyme Q in water is very low and depends directly on the length of the polyisoprene chain; increasing the chain length decreases the solubility in aqueous solvents.[6] The environment in which coenzyme Q is located influences spectral properties of the molecule and this has been exploited to monitor distribution of coenzyme Q in lipid/water systems. The polarity of the environment, for example, influences the ultraviolet spectral properties and this feature has been used to study the incorporation and partition of coenzyme Q into lipid bilayer dispersions and membranes.[7,8] Such studies have indicated that the environment of the chromophore is akin to that of a hydrocarbon such as iso-octane.

The solvation of coenzyme Q has been examined by the effect of different solvents on ^{1}H-NMR chemical shifts of particular residues.[9] It was found that the addition of dodecane to coenzyme Q causes a downfield shift of the entire proton resonance spectrum. Solvation by chloroform, on the other hand, indicates that the protons in the proximity of the benzoquinone substituent are affected differently compared to the remainder of proton resonances within the molecule. One of the remarkable features of solvation by chloroform is a significant chemical shift in proton resonances associated with the polyisoprene chain at low solvent concentrations. This effect was interpreted to indicate that the melt is not an isotropic liquid and that preferred associations between coenzyme Q molecules are preserved in the liquid phase. The solvation of the polar group may cause disruption of these associations and different regions of coenzyme Q are then able to interact. Another feature of solvation by chloroform is that the $-OCH_3$ resonances are split when the molar proportion of solvent to coenzyme Q reaches 5:1. This effect is not observed when coenzyme Q is solvated by dodecane. An important difference between the oxidized and reduced forms of coenzyme Q was that the $-OCH_3$ resonances of the reduced form were not split when solvated by chloroform.[10]

More precise information about how coenzyme Q partitions into membranes can be obtained by observing partitioning between phases of differing polarity. Experiments undertaken with ethanol-water solutions have shown that when the proportion of water in the solvent mixture exceeds about 10% by volume, there is a dramatic decrease in solubility of ubiquinone-10.[10] Examination of the precipitated material from ethanol/water mixtures by X-ray diffraction methods showed that there were no solvent molecules interposed between the isoprenoid chains of the ubiquinone in the ethanol/water phase.

An important consequence of the amphipathic character of coenzyme Q is its ability to orient as a monomolecular film at an air-water or oil-water interface. Studies of the monolayer properties of reduced and oxidized coenzyme Q at the air-water interface[11] indicate that stable monolayers are formed, which collapse at pressures considerably less than phospholipid monolayers. Compression isotherms of mixed monolayers of coenzyme Q and phospholipid indicate that the coenzyme Q molecules are squeezed out from between the phospholipid molecules at surface pressures well below the collapse pressure of the film. The coenzyme Q molecules form a layer on the top of the phospholipid monolayer. This effect is presumably due to the relatively weak polar interaction of coenzyme Q with water and consequently their tendency to leave the lipid-water interface.

2.3 INTERACTION OF COENZYME Q WITH MODEL MEMBRANES

Several strategies have been adopted to establish the location and orientation of coenzyme Q in membranes. Many of these studies have been performed with phospholipid bilayer model membranes on the assumption that coenzyme Q, being a lipid, will reside within the polar lipid bilayer matrix of membranes. Not all lipids in membranes, however, form bilayer structures when dispersed in dilute salt solutions at physiological temperatures. The most notable exceptions in animal cell membranes are the phosphatidylethanolamines and cardiolipins. Coenzyme Q itself is a membrane constituent, albeit in relatively low proportions, but as we have seen, it is largely insoluble in water and forms a separate hydrocarbon phase. The stability of the lipid bilayer matrix therefore depends on the balance maintained between the polar lipids of different amphipathic character and the manner in which they interact with each other and the membrane proteins. Two types of studies

have been performed, one aimed at determining how coenzyme Q interacts with other membrane lipids and the other to assess the effect of coenzyme Q on the stability of model membranes.

2.4 INCORPORATION OF COENZYME Q INTO PHOSPHOLIPID BILAYERS

The incorporation of coenzyme Q into bilayers of phosphatidylcholine and the effect that this has on the phase behavior of the mixture has been examined by differential scanning calorimetry. The method provides information on how the gel-to-liquid phase transition of the phospholipid is perturbed by the presence of coenzyme Q.[12–14] These studies have shown that codispersions of up to 20 mol% coenzyme Q_{10} in dipalmitoylphosphatidylcholine showed no significant affect on the temperature or enthalpy of the main gel-to-liquid crystalline phase transition or pretransition of the phospholipid. This is shown in Figure 2.1 which shows differential scanning calorimetric heating scans of phospholipid dispersions containing coenzyme Q_{10} or coenzyme Q_3. The scan of the dispersion containing 10 mol% coenzyme Q_{10} is almost identical to that of the pure phospholipid dispersion. In the presence of higher proportions of coenzyme Q_{10} thermotropic transitions of pure coenzyme Q_{10} are superimposed on that of the phospholipid indicating that the two components are phase separated. The inference from these data is that up to 20 mol% coenzyme Q_{10} in the phospholipid does not undergo normal thermotropic phase transitions nor does it affect the phase behavior of the phospholipid. These data are consistent with a location of coenzyme Q_{10} in the central hydrophobic domain of the bilayer.

2.5 EFFECT OF POLYISOPRENE CHAIN LENGTH

In contrast to coenzyme Q with relatively long polyisoprenoid substituents, molecular species with isoprenoid chains shorter than 5 cause significant changes in the phase behavior of the phospholipid in a manner suggesting that they are intercalated between the molecules of phospholipid in bilayer

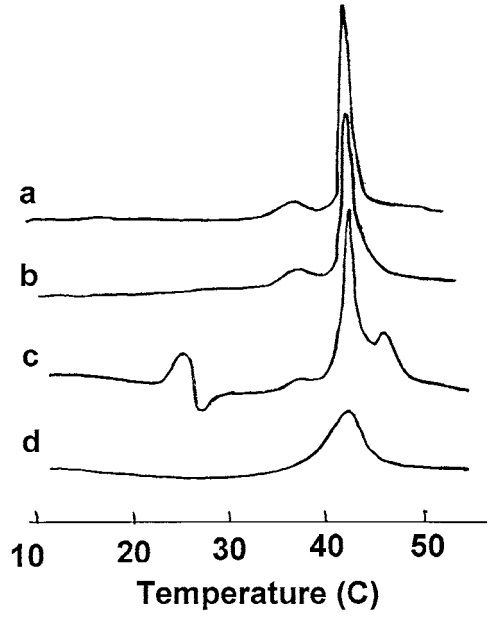

FIGURE 2.1 Differential scanning calorimetric heating curves of aqueous dispersions of dipalmitoylphosphatidylcholine (a) and phospholipid codispersed with (b) 10 mol% coenzyme Q_{10} (c) 50 mol% coenzyme Q_{10} (d) 4.5 mol% coenzyme Q_3.

configuration.[15] This can also be seen in Figure 2.1 where the presence of only 4.5 mol% coenzyme Q_3 apparently removes the pretransition endotherm and broadens out the main transition endotherm. The interpolation of coenzyme Q molecules with short polyisoprene chains between the phospholipid molecules is presumably dictated by the shift in amphipathic balance within the coenzyme Q molecule, which serves to anchor the benzoquinone moiety at the aqueous interface. Similar studies of ubiquinol-10 indicate that the reduced form of the coenzyme has a detectably greater affect on the enthalpy and cooperativity of phospholipid phase transitions, which argues for a more polar character of the reduced compared to the oxidized form of coenzyme Q.[16]

2.6 SPECTROSCOPIC STUDIES OF COENZYME Q–PHOSPHOLIPID MIXTURES

Proton nuclear magnetic resonance spectroscopic studies of coenzyme Q_{10} codispersed with phospholipid bilayers also have supported the notion that there is not extensive mixing of the two lipids in such arrangements.[10,17–19] ^{1}H-NMR spectra of coenzyme Q_{10} compared with multilamellar dispersions of phospholipid containing coenzyme Q_{10} show a high resolution spectrum of coenzyme Q_{10} superimposed over a broadened phospholipid spectrum. This is illustrated in Figure 2.2, which shows a spectrum of pure coenzyme Q_{10} in the isotropic melt compared with a multilamellar codisperson of 20 mol% coenzyme Q_{10} in dipalmitoylphosphatidylcholine recorded at 30°C. This temperature is below the gel-liquid crystalline phase transition temperature of the phospholipid and the melting point of the pure coenzyme Q_{10}. From the published peak assignments for proton resonances of coenzyme Q_{10},[20] it is found that there is some broadening of resonances in the isotropic liquid phase of coenzyme Q_{10}, which can be resolved when the coenzyme is dissolved in solvent as seen from a 200 MHz ^{1}H-NMR spectrum recorded from coenzyme Q_{10} dissolved in chloroform. The major difference between the proton spectrum of the melt and solution of coenzyme

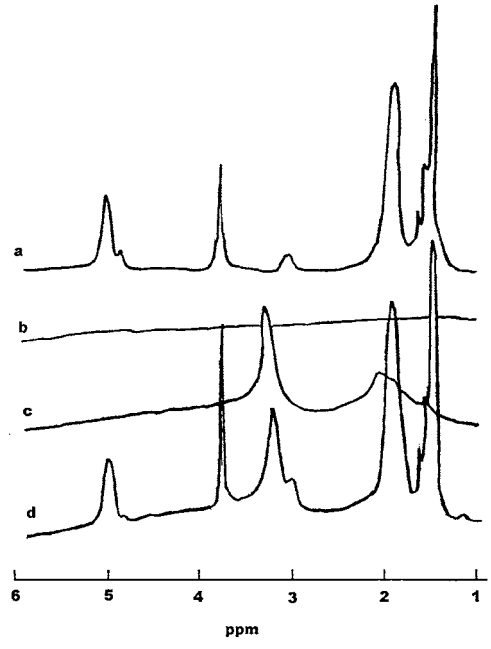

FIGURE 2.2 200 MHz ^{1}H-NMR spectra of (a) coenzyme Q_{10} recorded at 50°C and (b) at 30°C; (c) a multilamellar dispersion of dipalmitoylphosphatidylcholine in ^2H_2O recorded at 30°C; (d) a codispersion of 20 mol% coenzyme Q_{10} in dipalmitoylphosphatidylcholine in ^2H_2O recorded at 30°C.

Q_{10} is the chemical shift of the substituents of the benzoquinone ring. Thus, the relative chemical shifts of the $-OCH_3$ resonances differ by nearly 0.14 ppm when isoprene methyl protons in the two samples are assigned the same chemical shift. The resonances in the melt are upfield relative to the two resonances seen in chloroform, consistent with ring-current effects arising from a close proximity of the benzoquinone rings in the melt. The spectrum of the ubiquinone-10 codispersed with phospholipid was recorded below the phase transition temperature of the phospholipid and hence the proton resonances of this component are broadened and only those resonances from the choline group are readily resolved. Again, the relative chemical shift of the $-OCH_3$ protons of coenzyme Q_{10} in this codispersion indicates than the benzoquinone ring systems are in close proximity and small aggregates of coenzyme Q_{10} may be the most likely arrangement of these molecules in the dispersion. Alternatively, motion of groups within the molecules that take place independently of the whole molecule, could give rise to sharp peaks. All of the proton resonances of coenzyme Q_{10} seen in the melt that are resolved in the mixed phospholipid dispersion have similar relative intensities suggesting that motion of individual groups within the coenzyme Q molecule, when dispersed together with phospholipid, is unlikely. This is not the case with the phospholipid, in which the proton resonances of the hydrocarbon chains are broadened out and the residual choline methyl proton resonances located at 3.2 ppm have a relatively low intensity compared to that in multibilayer liposomes at temperatures greater than the gel-liquid crystalline phase transition temperature.[21] Spectra recorded at temperatures above the gel-to-liquid crystalline phase transition temperature of the phospholipid showed a marked increase in resonances associated with the hydrocarbon chains as well as the choline head group of the phospholipid. Moreover, the high-temperature studies indicated that the environment of the ubiquinone did not change on heating above the phase transition temperature of the phospholipid, since there was no evidence of any splitting or downfield shift of the $-OCH_3$ proton resonances.

Integration of the area under selected resonance peaks and comparison of these with corresponding resonances of coenzyme Q dissolved in chloroform provides an indication of the proportion of coenzyme Q that contributes to the high-resolution ^{1}H-NMR spectrum in mixed phospholipid dispersions. Such comparisons show that nearly all of the coenzyme Q in mixtures with synthetic phospholipids in ratios of 15 mol% or less contribute to the high-resolution signal. This proportion decreases as the amount of coenzyme Q in the mixture increases. With egg phosphatidylcholine mixtures, much lower proportions of coenzyme Q contribute to the signal. This result resembles the proportion of coenzyme Q that undergoes normal melting and crystallization in mixed dispersions as revealed by calorimetry.[13]

2.7 PHASE SEPARATION OF COENZYME Q IN MODEL MEMBRANES

It has been argued on the basis of measurements of transition enthalpy,[13] that virtually all of the phospholipid is phase-separated from coenzyme Q in mixed dispersions when the bilayer is in the gel state. This is not consistent with the proposal of Stidham et al.,[22] that the phospholipid partitions into a separate phase enriched in coenzyme Q. The model is also inconsistent with ^{31}P-NMR experiments, which show that virtually all of the phospholipid in the multibilayer dispersions of coenzyme Q with phospholipid are in a bilayer configuration and there is no evidence for a separate isotropic phase of phospholipid. Apart from differences in the relative motion of the coenzyme Q contributing to the high-resolution proton resonances and those of the phospholipid, the relative chemical shift of the $-OCH_3$ protons indicates that these groups still experience ring-current effects, but not to quite the same extent as in the melt. This suggests that although coenzyme Q is in a, so-called, "Q-rich phase," it is not in a crystalline form and possibly represents that proportion of the coenzyme Q in the system that is removed from the normal melting and crystallization typical of the unincorporated fraction.

The model that best fits these data is one in which coenzyme Q_{10} is integrated into phospholipid bilayers in a separate domain, which results in minimum perturbation of the phospholipid phase behavior. The coenzyme Q molecules interact with one another via stacking of the benzoquinone rings. The coenzyme Q experiences rapid motion and does not undergo crystallization at temperatures well below the melting point. The maximum amount of coenzyme Q that can be incorporated into phospholipid bilayers is about 20 mol%, and in mixed dispersions containing higher proportions, the excess coenzyme Q appears to behave as if it were in a separate phase of pure coenzyme Q.

2.8 SPECTROSCOPIC PROBE STUDIES OF COENZYME Q LOCATION

Further information about the location of the mobile pool of coenzyme Q in phospholipid dispersions has been obtained by the use of aqueous and lipophilic complexes of paramagnetic lanthanides on the proton resonances of the groups within the mixture.[20] The lanthanides used in these experiments were dysprosium in a water soluble nitrate salt and as an FOD complex. Studies in solution show a selective broadening and an upfield chemical shift of the protons of groups close to the benzoquinone ring. This region of the molecule represents the major polar domain within the coenzyme and there appears to be a preferential interaction with FOD complex of the lanthanide. Line broadening of proton resonances arising from the polyisoprenoid chain is comparatively small over a broad range of lanthanide concentrations. Similar experiments were undertaken on the effects of lanthanides on phospholipids in solution and, as with coenzyme Q, the line-broadened and chemically shifted proton resonances were restricted to groups located adjacent to the polar groups of the phospholipid, particularly those near the choline residue. With the knowledge of the effects of these lanthanide shift reagents on the two components of the system, it was possible to distinguish the effects of dysprosium located in the aqueous domain of multibilayer codispersions of coenzyme Q and egg phosphatidylcholine and dysprosium FOD confined to the hydrocarbon domain of the mixture. Such experiments showed that with increasing dysprosium FOD concentrations, there was a progressive broadening of the $-OCH_3$ proton resonances but only a slight perturbation of the choline methyl protons of the phospholipid. In the presence of dysprosium nitrate, however, line broadening of the choline methyl resonances was much more pronounced than with the FOD derivative. This suggests that the preferred location of the dysprosium nitrate is in the aqueous phase and dysprosium FOD is confined to the hydrophobic domain of the dispersion. There does not appear to be significant perturbation of the $-OCH_3$ proton resonances by the water soluble lanthanide, at least when present in concentrations of less than about 60 μM. This suggests that the benzoquinone substituent has a preferred location within the hydrophobic domain of the dispersion accessible to the dysprosium complex with FOD.

It has also been suggested on the basis of the effects of NMR chemical shift reagents on mixed dispersions of coenzyme Q and phospholipid,[23] that coenzyme Q_{10} is in rapid exchange across the bilayer walls of unilamellar phospholipid vesicles. Nevertheless, sonicated aqueous dispersions of coenzyme Q can form metastable aggregates that are small enough and sufficiently mobile to yield high-resolution ^{1}H-NMR spectra, which may be interpreted as coenzyme Q molecules rapidly migrating across the bilayer.[24] In other ^{1}H-NMR studies,[18] the possibility of rapid flip-flop motion of coenzyme Q across the bilayer of unilamellar vesicles was discounted and it was suggested that a large fraction of the coenzyme is located in a mobile pool near the center of the lipid bilayer.

Solid-state ^{13}C-NMR studies have also been used to determine the location of the benzoquinone ring system of coenzyme Q in phospholipid bilayers.[25] It was concluded that the polarity of the reduced form of coenzyme Q served to locate the ubiquinol ring closer to the lipid-water interface than was the case with the ubiquinone form, which tended to occupy a central core domain within the bilayer.

2.9 FLUORESCENCE PROBE STUDIES

Coenzyme Q in its oxidized form (ubiquinone), but not in its reduced state (ubiquinol), is an effective quencher of fluorescence of dyes. Localization of suitable chromophores at defined regions within phospholipid bilayer membranes can be used, in turn, to localize the benzoquinone moiety of coenzyme Q within the structure. This approach has been reported using anthroyloxy derivatives of stearic and palmitic acids incorporated into phospholipid bilayers.[26,27] Studies of the intensity of fluorescence quenching of these probes by a homologous series of coenzyme Q molecules differing in length of polyisoprene chain, has indicated that the benzoquinone substituent of shorter chain homologues like coenzyme Q_3 have access to all regions of the hydrocarbon domain of the bilayer. Longer chain homologues, by contrast, tend to be localized along a central plane of the bilayer where the terminal methyl groups of the phospholipid acyl chains reside. This localization is more apparent when the phospholipid is in the gel phase and greater penetration between the phospholipid molecules is observed when the bilayer is in a liquid-crystalline state.

The quenching of fluorescence of the mobile probe, diphenylhexatriene, provides more convincing evidence for changes in location of the benzoquinone residue on transition of the phospholipid from gel to liquid crystalline phase. Dynamic fluorescence quenching of membrane-bound dyes has led to the formulation of a model in which coenzyme Q undergoes translational diffusion in two dimensions within the membrane bilayer with the benzoquinone ring oscillating between the two bilayer surfaces within the hydrophobic domain, but not extending beyond the glycerol backbone of the phospholipid molecules anchored at the aqueous interface.

The changes in diffusional motion and domain formation induced by the presence of cholesterol measured by a fluorescence recovery after photobleaching method is the same for phospholipid as it is for a fluorescent derivative of coenzyme Q.[28,29] It was concluded from these dynamic studies that the coenzyme Q analogue does not preferentially reside at either the surface or the midplane of the phospholipid bilayer, but is highly mobile both laterally and transversely. The average residence localization is among the acyl chains of the phospholipid where it experiences the same microviscous environment and is affected in the same manner by the presence of cholesterol.

Measurements of polarization of fluorescence of diphenylhexatriene interpolated into phospholipid bilayers containing coenzyme Q_3 has provided information on the perturbation of the phospholipid bilayer caused by the presence of coenzyme Q.[30] The existence of two distinct populations of the probe were inferred, both of which could be quenched by the coenzyme. The dynamic motion of the probe indicated that the presence of coenzyme Q induced a small perturbation of the ordering of the acyl chains of the phospholipid molecules and a significant increase in the fluidity of the hydrocarbon domain of the structure.

Another approach to the study of the orientation of coenzyme Q in membranes is to examine the optical activity of the coenzyme Q chromophore when present in lipid bilayer membranes.[31] Such studies of homologues of coenzyme Q with different isoprene chain lengths distributed in lipid bilayers were intepreted to indicate that all ubiquinones with an isoprene side chain were oriented in a mid-plane of the bilayer with their benzoquinone headgroups oscillating transversly across the membrane and reaching the lipid-water interface.

2.10 VIBRATIONAL SPECTROSCOPIC STUDIES

Vibrational spectroscopy has also been a useful method of probing the location and environment of coenzyme Q in model phospholipid bilayer membranes. For example, Fourier transform infrared spectroscopy has proved very useful for investigating codispersions of coenzyme Q with phospholipids.[32] Other computer methods allowing spectral subtraction are equally effective in these studies.[33] Absorption bands corresponding to CH_2 antisymmetric and symmetric stretching modes can be used to determine *trans-gauche* isomerizations of the acyl chains of the phospholipids in bilayer conformation. Acyl chain packing and conformation also affects absorption bands associated with

CH_2 bending and scissoring modes. C−C stretching and low-frequency vibrational modes can be detected only by Raman spectroscopy and have also been used to assign phases to acyl chain domains.

Studies using the Fourier transform infrared spectroscopic method have shown that when reduced and oxidized forms of coenzyme Q are incorporated into multibilayer vesicles of dipalmitoylphosphatidylcholine, differences between the redox states of the coenzyme can be detected.[32] Changes observed in the bands corresponding to the CH_2 stretching and scissoring vibrations and of the C=O stretching mode of the phospholipid confirmed that the presence of up to 25 mol% of the oxidized form of coenzyme Q_{10} does not have a marked effect on the phase transition of the phospholipid. Reduced coenzyme Q_{10} at the same concentration, by contrast, significantly alters the phase transition of the phospholipid causing a decrease of several degrees in the gel-to-liquid crystalline phase transition temperature and a broadening of the transition. It was suggested that the effects were due to a different localization of the oxidized and reduced forms of the coenzyme in the bilayer membrane.

Localization of coenzyme Q is exemplified by the data presented in Figure 2.3, which shows the infrared absorption spectra in the region of the CH stretching vibrations from which the signal obtained from water has been subtracted. Difference infrared spectra were recorded at 20°C (gel phase) and at 54°C (liquid crystal phase) and two strong bands located at 2920 and 2851 cm^{-1} are observed. These bands arise from the CH_2 antisymmetric and symmetric CH stretching modes of the fatty acyl chains respectively. Both these absorbances are sensitive to the change in phase of the phospholipid and become broader and weaker in intensity and shift to higher wave numbers above the phase transition temperature. This shift is apparent from the different spectrum obtained by subtraction of spectrum (a) from spectrum (b) in Figure 2.3. The thermal changes in the spectrum are believed to be due to the decrease in all-*trans* conformers and a corresponding increase in the *gauche* rotameters with increasing temperature. The line broadening of these bands is related to the increase in rates and amplitude of motion of the fatty acyl chains with increasing temperature.[34] Identical spectra were obtained from dispersions of phospholipid without coenzyme Q suggesting that there was no significant effect of coenzyme Q on acyl chains either above or below the phase transition of the phospholipid.

Two other absorption bands centerd at 2956 and 2872 cm^{-1}, attributed to CH antisymmetric and symmetric stretching vibrations, respectively, of the terminal methyl groups of the hydrocarbon chains of the phospholipid, are also resolved in the spectra shown in Figure 2.3. The band at 2956 cm^{-1} is the more prominent of the two, but, as indicated from the difference spectrum (spectrum c),

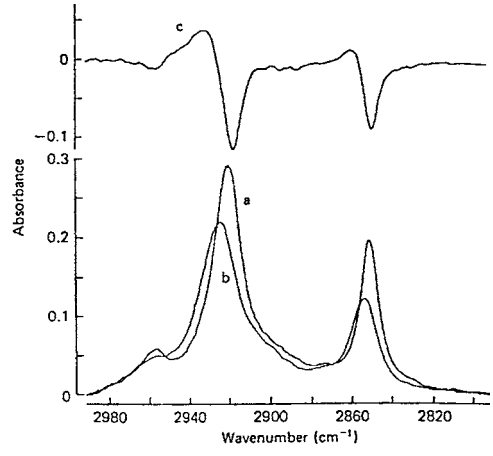

FIGURE 2.3 Infrared absorption spectra in the C–H stretching region of fully hydrated dipalmitoylphosphatidylcholine bilayers containing 5 mol% coenzyme Q_{10}. Spectra recorded at (a) 20°C; (b) 54°C. Spectrum (c) is a representative difference spectrum (a − b).

the intensity decreases when the dispersion is heated above the gel-liquid crystalline phase-transition temperature. Similar difference spectra of dispersions of pure phospholipid or phospholipid containing 15 mol% did not indicate that the presence of ubiquinone caused perturbation of the hydrocarbon chains as judged by either C–H stretching of the chain methylene or terminal methyl groups. An additional broad band centered around 2990 cm^{-1} and attributed to a weak Fermi resonance interaction between the symmetric methylene stretching mode and the first overtone of the methylene scissoring mode can also be seen in the scan recorded at higher temperature. The behavior of this absorbance also appears to be unperturbed by the presence of up to 15 mol% coenzyme Q in the dispersion.

In order to obtain more precise information about the effect of coenzyme Q on the pretransition and the main lamellar gel-to-liquid crystalline phase transition of the phospholipid, the temperature dependence of the frequency of maximum absorbance of the CH_2 antisymmetric C–H stretching vibrations have been determined.[35] It was found that the presence of 15 mol% coenzyme Q caused a 1 to 2°C decrease in the temperature of the midpoint of the change in frequency of maximum absorption associated with the main gel-liquid crystalline phase transition. Experiments using lower proportions (2.5 mol% coenzyme Q) showed approximately the same decrease in temperature of this parameter. There was no evidence of any differences in the temperature range of the pretransition of the phospholipid. Furthermore, the inflection in the spectrum seen in the lipid at temperatures between 20 and 25°C, which correlates with a factor group splitting effect observed in the CH_2 rocking mode,[36] does appear to be modified by the presence of coenzyme Q as judged by the temperature dependence of the half bandwidth of the CH_2 antisymmetric CH stretching vibration.

Absorption by the benzoquinone substituent of coenzyme Q was detected by spectral subtraction of dispersions of phospholipid from mixed dispersions of phospholipid and ubiquinone.[33] Bands due to C=O stretching and ester group vibrations of coenzyme Q in codispersion with phospholipid were compared with the same spectral region when coenzyme Q was dissolved in solvents such as chloroform or dodecane or in the crystalline solid. Differences were observed in the carbonyl conjugated C=C stretch band with solvent environment compared with the pattern observed in the same solvent systems on the C=O stretch band. The frequency of maximum absorbance of this band in coenzyme Q dispersed in phospholipid shifts to 1613.6 cm^{-1} when dissolved in chloroform with some decrease in intensity, and to 1613.4 cm^{-1} in dodecane without loss in intensity. In crystalline ubiquinone, this band is centred at 1610.6 cm^{-1}. Studies have also been undertaken in absorption bands centerd at 1088.9 and 1222.8 cm^{-1}, which are assigned to symmetric and antisymmetric PO_2-stretch vibrations, respectively, of the phospholipid polar head group. Difference spectra of phospholipid containing 15 mol% coenzyme Q compared with the phospholipid dispersion alone indicates that neither of the two PO_2-stretch vibrations are perturbed by the presence of coenzyme Q.

Analysis of the Fourier transform infrared spectra of coenzyme Q_{10} in phospholipid bilayers and detergent micelles has been undertaken by Castresana et al.[36] They reported that the C=O stretching band, which is relatively sensitive to the polarity of its environment, was invariably located at around 1665.5 to 1667.4 cm^{-1} irrespective of the physical state of the lipid, and suggested that coenzyme Q existed in a form similar to that of the pure melted compound. From these observations it was concluded that coenzyme Q is located in a hydrophobic environment within phospholipid bilayers in the form of phase-separated aggregates. The aggregates are believed to be interspersed among the hydrocarbon chains of the phospholipids and of a size smaller than that capable of representing a cooperative unit that could crystallize at temperatures lower than the melting temperature of pure coenzyme Q.

Bands with frequencies around 1262 cm^{-1} have been variously ascribed to C−O stretching of the methoxy groups of the benzoquinone ring or to −C−O− vibrations, both associated with the quinone function.[37–39] Bands in the region of 1550 to 1750 cm^{-1} and 1200 to 1350 cm^{-1} of the spectrum of quinones, and which are distinct from carbonyl bands and of higher intensity than in the corresponding spectra of related hydrocarbons, are generally attributed to coupling of the

carbonyl stretching with ring skeletal or other vibrational modes in the rest of the molecule.[40] For this reason, unequivocal assignments are not possible for these absorption bands in coenzyme Q, and it has been suggested that the larger contribution to the band near 1260 cm^{-1} is a quinanoid absorption that masks the methoxy band.[41] Other bands in this region can, however, be assigned more precisely to the $-O-CH_3$ group vibrations at around 1202, 1151, and 1101 cm^{-1},[42] but they are overlapped in this spectrum by the stronger phosphate and ester modes arising from the phospholipid. The origin of the peaks observed at 1072.7 and 1057.6 cm^{-1} is unknown, and no bands in this range have been assigned in the spectrum of coenzyme Q, although their intensity is directly related to the proportion of ubiquinone in the mixture.

Infrared absorption bands of polar bonds in the spectral region 1315 to 1240 cm^{-1} are known to be influenced by changes in the character of their environment. Spectral changes in this region associated with coenzyme Q in phospholipid compared with those in solvents suggest that the benzoquinone ring system is located within a hydrocarbon domain in dispersions with phospholipid. There is also an indication from the infrared studies that in codispersions of coenzyme Q with dipalmitoylphosphatidylcholine, the two lipids phase-separate within the system. Furthermore, there is no evidence that coenzyme Q intercalates between phospholipid molecules, which appear to undergo a gel-to-liquid crystalline phase transition in only a slightly modified form in the presence of coenzyme Q.

2.11 INTERACTION OF COENZYME Q WITH NONBILAYER FORMING PHOSPHOLIPIDS

Mixtures of coenzyme Q with nonbilayer forming lipids is particularly informative for assessing its influence on the stability of membranes. Membrane lipids like phosphatidylethanolamines form so-called hexagonal-II structures in which the bilayer is replaced by a three-dimensional structure consisting of phospholipid molecules arranged into tubes packed into a hexagonal array. The tendency of the lipids to form such structures is a function of the amphipathic balance within the molecule. Phosphatidylethanolamines are characterized by low hydration of the polar group, thereby shifting the amphipathic balance toward hydrophobic affinity. In mixtures with other hydrophobic molecules, aqueous dispersions of phosphatidylethanolamines are induced to form hexagonal-II phases at lower temperatures than that of the pure phospholipid.

Recent biophysical studies of codispersions of coenzyme Q with phosphatidylethanolamine have been reported, which indicate that coenzyme Q has a marked influence on the lamellar-to-hexagonal-II phase transition of the phospholipid.[43] Differential scanning calorimetric studies showed that, like bilayer-forming lipids, coenzyme Q had no significant effect on the temperature, cooperativity, or enthalpy of the gel-to-liquid crystalline phase transition of phosphatidylethanolamine, even when present in proportion of 50 mol%. There was, however, a marked decrease in the temperature of the lamellar-to-hexagonal-II phase transition and reduced coenzyme Q had a more perturbing effect than the oxidized form. The presence of an isotropic phase was identified by ^{31}P-NMR in codispersions containing 50 mol% of reduced, but not oxidized, coenzyme Q at higher temperatures. Phase separation of a coenzyme Q-rich phase was suggested from small-angle x-ray diffraction studies of these mixtures.

2.12 ARRANGEMENT OF COENZYME Q IN BIOLOGICAL MEMBRANES

Fluorescence quenching methods have been employed to locate the domain occupied by coenzyme Q in mitochondrial and other energy transducing membranes. One of the earliest reports was the use of anthroyloxy fatty acids to characterize changes in the oxidation state of mitochondria by monitoring changes in fluorescence induced by collision with the reduced coenzyme Q.[44] It was

reported that access of the oxidized ubiquinone in membranes depleted of substrate required a structural change in the membrane before it could enter the lipid pool probed by the dye. More detailed analysis using homologues of the anthroyloxy fatty acids with the dye attached at different positions along the hydrocarbon chain was undertaken to characterize the transverse organization of ubiquinone in heart mitochondria.[45] The results were intepreted to indicate the existence of two transverse positions of the benzoquinone ring, which was consistent with the presence of more than one spacially compartmentalized pool of ubiquinone in the inner mitochondrial membrane. The exchange between the different pools was slow relative to the ns timescale of the fluorescence quenching process.

Studies of nuclear magnetic resonance spectroscopic signals from deuterated derivatives of ubiquinone-10 interpolated into inner mitochondrial membranes and into the membrane of *Escherichia coli* indicate, in contrast to the fluorescence probe studies, only a single isotropically mobile pool of ubiquinone in these membranes.[24] The motion was apparently unhindered by orientational constraints imposed by the lipid bilayer matrix of the mitochondrial membrane. This was contrary to the findings with a shorter chain deuterated homologue, which did show orientational constraints that were consistent with an intercalation of the ubiquinone homologue between the membrane phospholipids. In studies of the restoration of the rate of electron transfer in membranes depleted of ubiquinone or mutants devoid of endogenous ubiquinone, no difference in rate between short and long chain ubiquinones was observed. This suggests that the respective domains occupied by the homologues are integrated into the redox span linked by coenzyme Q.

Lateral diffusion rates of a fluorescent ubiquinone analogue have been reported in fused, matrix-free inner mitochondrial membrane preparations and mitoplasts using fluorescence recovery after photobleaching methods.[46,47] Rates of lateral diffusion of the analogue measured at 23°C were in the order of 2.3 to $9.4.10^{-9}$ cm^2s^{-1} and showed no significant dependence on matrix density or extent of folding of the membrane. The results were consistent with the participation of coenzyme Q in random collisions with mitochondrial dehydrogenases and Complex III of the inner mitochondrial membrane.

2.13 CONCLUSIONS

The consensus model that emerges from the considerable body of data derived from biophysical studies of coenzyme Q in model and biological membranes is presented in the molecular model shown in Figure 2.4. Coenzyme Q homologues found in biological membranes have polyisoprene chain lengths greater than 5 units and commonly 9 to 10 units. This highly hydrophobic substituent of the molecule combined with the relatively weak polar groups located on the fully substituted

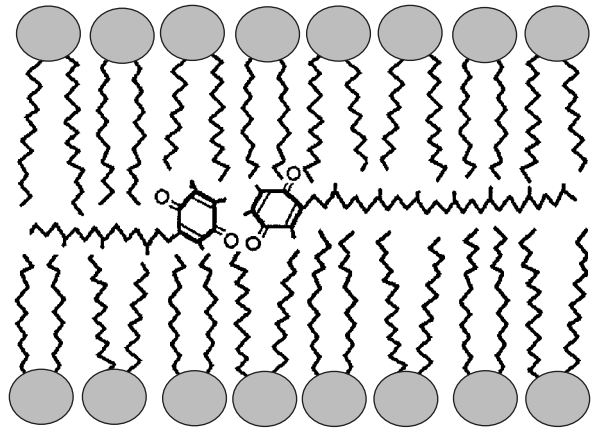

FIGURE 2.4 Schematic molecular model of the location of coenzyme Q in the membrane lipid bilayer matrix.

benzoquinone ring system renders the molecule more nonpolar than most of the lipids found in membranes. Thus the forces that tend to anchor the polar group of coenzyme Q at the lipid-water interface are weak and are counterbalanced by the extensive nonpolar group of the molecule.

These thermodynamic arguments provide an explanation of the results and conclusions drawn from the experimental evidence discussed in this chapter. Coenzyme Q can be seen in the model to occupy a domain in the hydrophobic core at the center of the lipid bilayer matrix. The molecules associate into clusters of relatively few molecules. Interactions occur between the benzoquinone rings, which may associate via van der Waals forces arising from electron delocalizations about the rings. The clusters are mobile within the lipid matrix and can diffuse laterally in the plane of the membrane and rotate about an axis parallel to the plane of the bilayer. The reduced form of coenzyme Q has a more polar character than its oxidized counterpart. This difference in polarity allows the reduced form of coenzyme Q to penetrate closer to the lipid-water interface than the oxidized form, which remains constrained more to the central hydrophobic domain.

It is acknowledged that this is a comparatively simple model and does not take into account the interaction of coenzyme Q with specific Q-binding proteins and redox centers. Nor does the model consider the conformation of the polyisoprenoid chain, which may be coiled rather than fully extended as dicussed in Chapter 1. Nevertheless, the model provides a basis upon which more rigorous tests can be applied.

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3 Free Radical Chemistry of Coenzyme Q

Kazuo Mukai

CONTENTS

3.1 INTRODUCTION

In recent years, lipid peroxidation has attracted much attention because it induces biomembrane destruction and is related to inflammation, heart disease, cancer, and even aging. Vitamin E (tocopherol, TocH) is localized in cellular membranes and functions as an antioxidant by protecting unsaturated lipids from peroxidation.[1,2] Vitamin E reacts with lipid peroxyl radical (LOO•) and reduces it to hydroperoxide (LOOH). Vitamin E itself is oxidized and produces vitamin E radical (Reaction 1).[3,4]

$$\mathrm{LOO\bullet} + \mathrm{TocH} \xrightarrow{k_{\mathrm{inh}}} \mathrm{LOOH} + \mathrm{Toc\bullet} \tag{3.1}$$

On the other hand, hydrophilic vitamin C [ascorbate monoanion, (AsH^-)] is, by itself, a poor antioxidant, but it enhances the antioxidant activity of tocopherols by regenerating the tocopheroxyl to tocopherol (Reaction 2).[1,2,5]

$$\mathrm{Toc\bullet} + \mathrm{AsH^-} \xrightarrow{k_r} \mathrm{TocH} + \mathrm{A\tilde{s}\bullet} \tag{3.2}$$

where $A\tilde{s}\bullet$ is dehydroascorbate anion radical.

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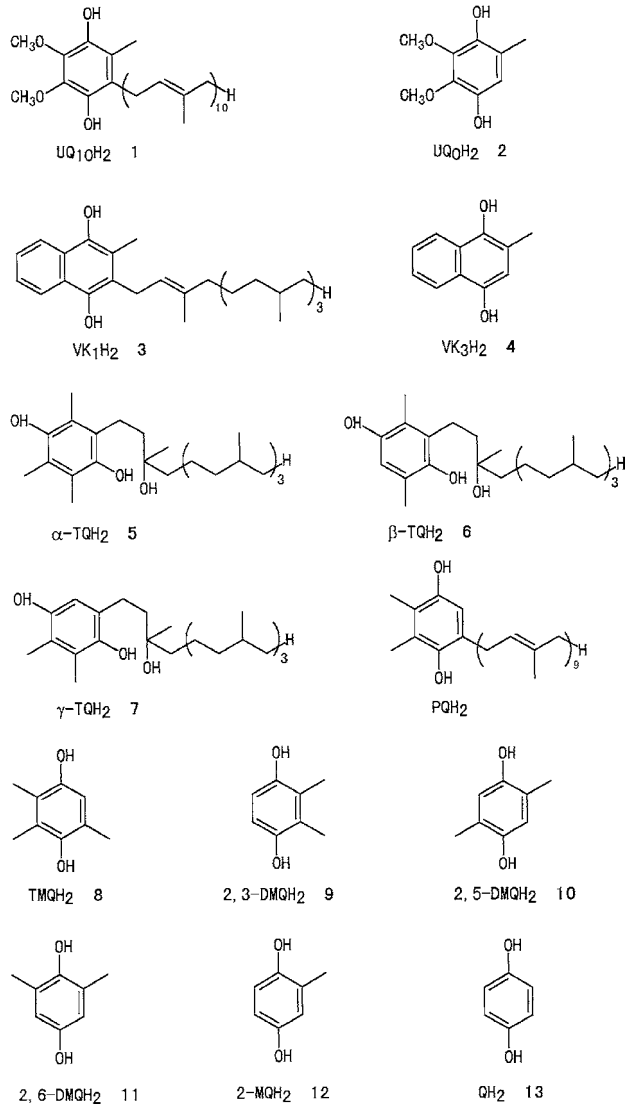


FIGURE 3.1 Molecular structures of biological hydroquinones **1–7**, plastoquinol (PQH_2), and related hydroquinones **8–13**.

Ubiquinone (UQ), vitamin K (VK), and plastoquinone (PQ) are well known as typical biological quinone compounds. The function common to these quinones in biology is to act as redox components of transmembrane electron transport systems. Ubiquinol (UQH_2), vitamin K hydroquinone (VKH_2), and plastoquinol (PQH_2) (Figure 3.1) are the two-electron reduction products of UQ, VK, and PQ, respectively.

Several investigators have found that ubiquinol has a strong activity in inhibiting lipid peroxidation in various tissues and membranes.[6–9] Further, it has been reported that, in membranes, ubiquinols with short isoprenoid chains (ubiquinol-1-4) are much more potent inhibitors of lipid peroxidation than the longer chain homologues (ubiquinol-5-10).[10,11] It has been suggested that ubiquinol (UQH_2) functions as an antioxidant (i) by scavenging the lipid peroxyl radical (LOO•) (Reaction 3), and (ii) by regenerating the tocopheroxyl to tocopherol

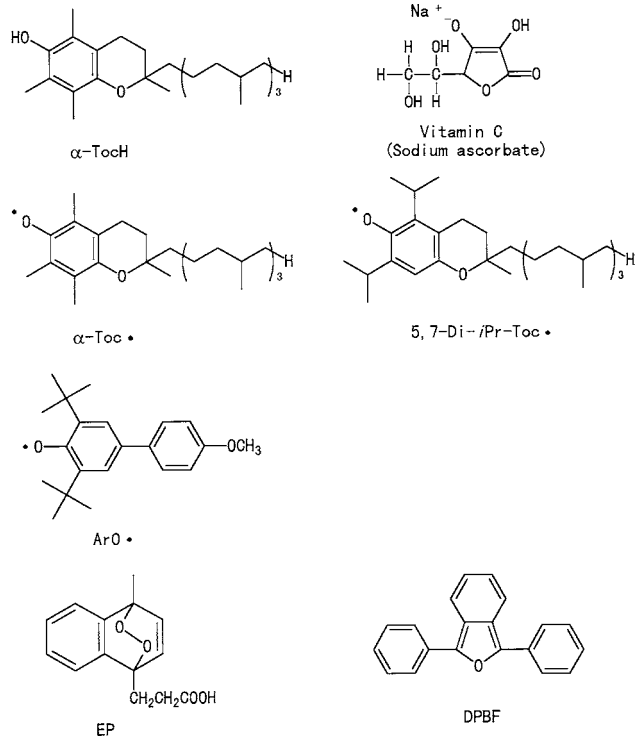


FIGURE 3.2 Molecular structures of α-tocopherol (α-TocH), sodium ascorbate (Na^+AsH^-), α-tocopheroxyl (α-Toc•), 5,7-diisopropyltocopheroxyl (5,7-Di-*i*Pr-Toc•), aroxyl radical (ArO•), EP, and DPBF.

(Reaction 4),[7,10,12–15]

$$\mathrm{LOO\bullet} + \mathrm{UQH_2} \xrightarrow{k_{\mathrm{inh}}} \mathrm{LOOH} + \mathrm{UQH\bullet} \tag{3.3}$$

$$\mathrm{Toc\bullet} + \mathrm{UQH_2} \xrightarrow{k_r} \mathrm{TocH} + \mathrm{UQH\bullet} \tag{3.4}$$

where UQH• is the dehydroubiquinol radical. However, the antioxidant activities of the biological hydroquinones, except for ubiquinol, have not been reported.

In previous works, we measured the reaction rates, k_S, of α-, β-, γ-, and δ-tocopherols (α-, β-, γ-, and δ-TocHs) with 2,6-di-tert-butyl-4-(4-methoxyphenyl)-phenoxyl [ArO• (abbreviated to "aroxyl" hereinafter), Figure 3.2] in ethanol solution (Reaction 5), using stopped-flow spectrophotometry.[16,17] ArO• can be regarded as a model of active oxygen radical (LOO•, LO•, and HO•) in biological systems.

$$\mathrm{ArO\bullet} + \mathrm{TocH} \xrightarrow{k_S} \mathrm{ArOH} + \mathrm{Toc\bullet} \tag{3.5}$$

The second-order rate constants, k_S, obtained are 5.12×10^3 (α-TocH), 2.24×10^3 (β-TocH), 2.42×10^3 (γ-TocH), and 1.00×10^3 (δ-TocH) $M^{-1}s^{-1}$ in ethanol at 25.0°C. The relative rates (α:β:γ:δ = 100:44:47:20) agree well with those obtained from studies of the reactivities of tocopherols toward poly(peroxystyryl)peroxyl radicals (100:41:44:14) by the O_2 consumption method (Reaction 1).[3,18]

The result suggests that the relative reactivities of tocopherols in solution probably do not depend on the kinds of oxyradicals (ArO• and LOO•) used.[17]

Lipid peroxidation is also induced by the reaction of singlet oxygen ($^1O_2$) with unsaturated lipids. Tocopherols can also act as efficient scavengers of singlet oxygen.[19–24] It was shown that α-tocopherol scavenges $^1O_2$ by a combination of physical quenching (k_q) and chemical reaction (k_{cr}). Because $k_q >> k_{cr}$, the quenching process is almost entirely "physical"; that is, α-tocopherol deactivates about 120 $^1O_2$ molecules before being destroyed by chemical reaction (Reaction 6):[19]

$$^1O_2 + \text{TocH} \xrightarrow{k_Q} \text{physical quenching} + \text{chemical reaction} \tag{3.6}$$

It has been reported that ubiquinol quenches hematoporphyrin-activated singlet oxygen in mitochondria.[25] However, the kinetic study of the quenching reaction of singlet oxygen by biological hydroquinones has not been reported.

Therefore, in order to clear the antioxidant activity of these biological hydroquinones (HQs), systematic kinetic studies of Reactions 3, 4, 5, and 6 have been performed for eight HQs (ubiquinol-10 ($UQ_{10}H_2$ **1**); ubiquinol-0 (UQ_0H_2 **2**); vitamin K_1 HQ (VK_1H_2 **3**); vitamin K_3 HQ (VK_3H_2 **4**); α-, β-, and γ-tocopoherol-HQs (α-, β-, and γ-TQH_2 **5–7**); and 2,3,5-trimethyl-1, 4-HQ ($TMQH_2$ **8**) (Figure 3.1) in several solutions. The observed rates, k_{inh}, k_r, k_S, and k_Q, were compared to those of the α-tocopherol and vitamin C, which are well known as important biological antioxidants.

3.2 EXPERIMENTAL PROCEDURES

Preparations of seven biological hydroquinones (**1–7**) and related hydroquinones (**8–13**) (Figure 3.1) are reported in previous papers.[26,27] 3-(1, 4-Epidioxy-4-methyl-l,4-dihydro-1-naphthyl) propionic acid (EP, Figure 3.2) was prepared by the published procedure.[28] 2,5-Diphenyl-3, 4-benzofuran (DPBF) is commercially available. The aroxyl radical (2,6-di-tert-butyl-4-(4-methoxyphenyl) phenoxyl) (ArO•, Figure 3.2) was prepared according to the method of Rieker and Scheffler.[29]

The 5,7-diisopropyltocopheroxyl radical (5,7-Di-*i*Pr-Toc•, Figure 3.2) is fairly stable and was prepared by PbO_2 oxidation of the corresponding tocopherol in ethanol, benzene, and isopropyl alcohol/water (5:1, v/v) solutions in a nitrogen atmosphere. However, in the case of α-tocopherol (α-TocH), the α-tocopheroxyl radical (α-Toc•) produced is unstable, and the absorption spectrum decreases rapidly with time. Therefore, the α-tocopheroxyl radical was prepared by the reaction between the stable aroxyl radical (ArO•) and α-tocopherol in ethanol, benzene, and isopropyl alcohol/water (5:1, v/v) solutions at 25 °C under nitrogen atmosphere and was reacted immediately with biological hydroquinone solution.[26]

The stopped-flow data were obtained on a UNISOKU Model RS-450 stopped-flow spectrophotometer (Osaka, Japan) by mixing equal volumes of solutions of the tocopheroxyl radical (Toc•) [or aroxyl radical (ArO•)] and biological hydroquinone.[26] The oxidation reactions were studied under pseudo first-order conditions, and the observed rate constants (k_{obsd}) were calculated in the usual way using a standard least-square analysis. All measurements were performed at 25.0 ± 0.1°C.

Cyclic voltammetry was performed at 25°C under an atmosphere of nitrogen with a platinum electrode and a saturated calomel reference electrode in acetonitrile (dried over P_2O_5) containing 40 mM tetrabutylammonium perchlorate with a Yanaco Model P-1000 H cyclic voltammetric analyzer. Under these conditions, ferrocene as a standard sample has a half-wave potential of +400 mV. The observed E_P values are summarized in Tables 3.4 and 3.5. The experimental error in E_P values was ±20 mV at maximum.

3.3 FREE-RADICAL-SCAVENGING ACTION OF BIOLOGICAL HYDROQUINONES

3.3.1 Kinetic Study of Aroxyl-Radical-Scavenging Action of Biological Hydroquinones in Solution

The oxidation rates of biological hydroquinones (HQs **1–8**) by aroxyl (ArO•) were studied spectrophotometrically by the stopped-flow technique in the presence of excess hydroquinone in ethanol solution (Reaction 7). The details of these experiments are reported in a previous paper.[16,30]

$$\mathrm{ArO\bullet} + \mathrm{HQ} \xrightarrow{k_S} \mathrm{ArOH} + \mathrm{Q\bullet} \tag{3.7}$$

The rate was measured by following the decrease in absorbance at 375 and/or 580 nm of the ArO• radical. The pseudo-first-order rate constants, k_{obsd}, observed at 375 and/or 580 nm were linearly dependent on the concentration of hydroquinone (HQ), and thus the rate equation is expressed as

$$-d[\mathrm{ArO\bullet}]/dt = k_{obsd}[\mathrm{ArO\bullet}] = k_S[\mathrm{HQ}][\mathrm{ArO\bullet}] \tag{3.8}$$

where k_S is the second-order rate constant for oxidation of HQ by ArO• radical. The rate constants, k_S, were obtained by plotting k_{obsd} against [HQ], as shown in Figure 3.3.

Similar measurements were performed for the reaction of ArO• with HQs **1–8** in diethyl ether, benzene, and *n*-hexane solution. The values of k_S obtained are listed in Table 3.1, together with that of α-tocopherol.[30] The experimental error in the k_S value for each hydroquinone was ~7% at maximum. The radical-scavenging rate constants, k_S, of vitamin K_1 and vitamin K_3 hydroquinones were very fast in *n*-hexane solution, and we could not succeed in determining the rate constants, k_S, for these hydroquinones. We estimated the lowest values of rate constant, k_S, of VK_1H_2 and VK_3H_2 to be about 10^7 $M^{-1}s^{-1}$. β-TQH_2 **6** and $TMQH_2$ **8** were insoluble in *n*-hexane. On the other hand, by reacting aroxyl with biological quinones **1–8** in ethanol, the decrease of absorption at 376 nm of ArO• was negligible. The result indicates that hydroquinones act as free radical scavengers by donating a hydrogen atom of the 1-OH and/or 4-OH group to the aroxyl radical. As listed in Table 3.1,

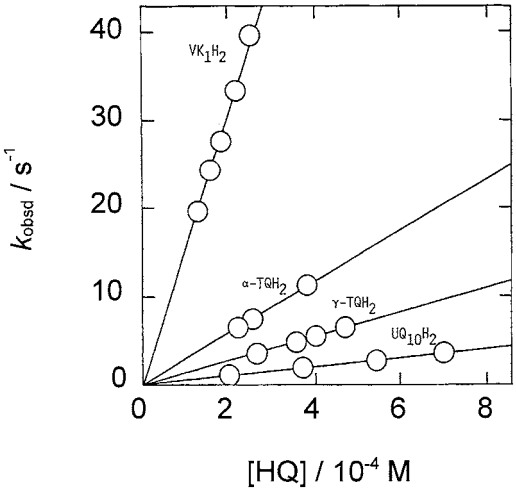


FIGURE 3.3 The dependence of the pseudo-first-order rate constant, k_{obsd}, on the concentration of biological hydroquinones ($UQ_{10}H_2$ **1**, VK_1H_2 **3**, α-TQH_2 **5**, and γ-TQH_2 **7**) in ethanol solution.

TABLE 3.1
Second-Order Rate Constants (k_S) and Relative Rate Constants (k_S (HQ)/k_S (α-TocH)) for the Reaction of Aroxyl Radical (ArO•) with Biological Hydroquinones 1–8 in Several Solvents at 25.0°C

	Ethanol		Diethyl Ether		Benzene		*n*-Hexane	
	k_s ($M^{-1}s^{-1}$)	k_s (HQ)/k_s(α-TocH)	k_s ($M^{-1}s^{-1}$)	k_s (HQ)/k_s(α-TocH)	k_s ($M^{-1}s^{-1}$)	k_s (HQ)/k_s(α-TocH)	k_s ($M^{-1}s^{-1}$)	k_s (HQ)/k_s(α-TocH)
α-TocH	5.12×10^3	1.00	1.44×10^4	1.00	9.52×10^4	1.00	1.94×10^5	1.00
$UQ_{10}H_2$ **1**	5.19×10^3	1.01	3.89×10^3	0.27	1.14×10^4	0.12	2.11×10^4	0.11
UQ_0H_2 **2**	2.90×10^3	0.57	2.65×10^3	0.18	1.30×10^4	0.14	2.00×10^4	0.10
VK_1H_2 **3**	1.61×10^5	31.4	2.31×10^5	16.0	1.53×10^6	16.1	$>10^7$	
VK_3H_2 **4**	1.07×10^5	20.9	1.54×10^4	10.7	1.80×10^6	18.9	$>10^7$	
α-TQH_2 **5**	1.40×10^4	2.73	2.92×10^4	2.03	1.27×10^5	1.33	1.45×10^5	0.75
β-TQH_2 **6**	8.35×10^3	1.63	1.54×10^4	1.07	1.26×10^5	1.32	Insoluble	–
γ-TQH_2 **7** (PQH_2 model)	1.46×10^4	2.85	3.65×10^4	2.53	2.15×10^5	2.26	7.60×10^5	3.92
$TMQH_2$ **8**	8.62×10^3	1.68	2.20×10^4	1.53	2.27×10^5	2.38	Insoluble	–

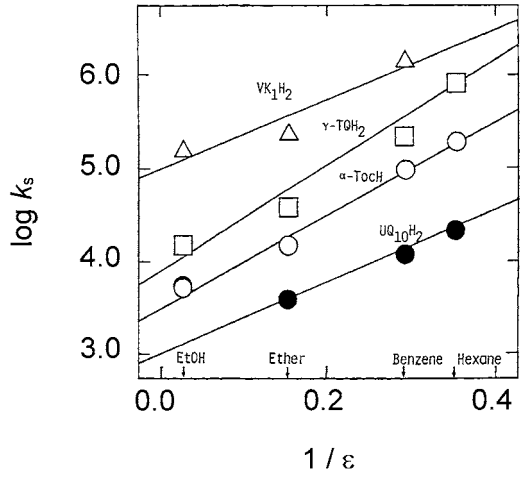


FIGURE 3.4 Plot of log k_S vs. 1/ϵ for biological hydroquinones ($UQ_{10}H_2$ **1**, VK_1H_2 **3**, and γ-TQH_2 **7**) and α-tocopherol (α-TocH). On a line, each point corresponds to ethanol, diethyl ether, benzene, and *n*-hexane from left to right.

the rate constant of $UQ_{10}H_2$ **1** is very similar to that of α-TocH in ethanol solution. On the other hand, $UQ_{10}H_2$ **2** is 0.57-fold as reactive as α-TocH. However, α-TQH_2 **5**, β-TQH_2 **6**, γ-TQH_2 **7**, and $TMQH_2$ **8** were found to be 2.73, 1.63, 2.85, and 1.68-fold more reactive than the α-TocH in ethanol, respectively. Further, VK_1H_2 **3** and VK_3H_2 **4** were found to be 31.4 and 20.9-fold more reactive than α-TocH, which has the highest reactivity among natural tocopherols.[3,18] Consequently, these vitamin K hydroquinones have the highest free-radical-scavenging (FRS) activity among natural lipid-soluble, chain breaking antioxidants in solution.[30–32]

In each hydroquinone, the FRS rate constant, k_S, increased by decreasing the polarity of solvent. When the logarithm of the rate constant (log k_S) of γ-TQH_2 **7** was plotted as a function of the reciprocal of the solvent dielectric constants (1/ϵ), it gave a straight line (Figure 3.4).[30] The same linear relationship between the log k_S and the 1/ϵ was also obtained for VK_1H_2, α-TocH, and $UQ_{10}H_2$, except for the value of $UQ_{10}H_2$ in ethanol, as shown in Figure 3.4. The HQs **3–8** showed

higher reactivity than α-TocH regardless of the polarity of the solvent. In polar ethanol solvent, the order of magnitude of k_S value was **3** > **4** > **5** > **7** > **6** ~ **8** > α-TocH ~**1** > **2**. On the other hand, in nonpolar benzene solvent, the order was **4** > **3** > **8** > **7** > **5** ~ **6** > α-TocH > **2** > **1**. The result indicates that the approximate order of magnitude of the scavenging rate of free radical by biological HQs are (i) VK_1H_2 and VK_3H_2 > (ii) α-, β-, γ-TQH_2 and $TMQH_2$ > (iii) α-TocH > (iv) $UQ_{10}H_2$ and $UQ_{10}H_2$ in solution.

As reported in previous papers, absolute reactivities of tocopherols to ArO• and LOO• (k_S and k_{inh}, respectively) increase as the total electron-donating capacity of the alkyl substituents on the aromatic ring increases.[33] In fact, as listed in Table 3.1, the k_S values of β-TQH_2, γ-TQH_2, and $TMQH_2$ are similar to each other in solution, because these HQs have three alkyl substituents on the aromatic ring. Plastoquinone, which is very important as an electron carrier in photosynthetic systems, also has three alkyl substituents on the aromatic ring, and we can expect that the FRS rate of plastoquinol (PQH_2) is similar to those of β-TQH_2, γ-TQH_2, and $TMQH_2$ in solution. Especially, both the PQH_2 and γ-TQH_2 have two methyl substituents at 2- and 3-positions and a long-alkyl-chain at 6-position, and thus the reactivities of PQH_2 and γ-TQH_2 is thought to be similar. The result suggests that PQH_2 also scavenges the active oxygen free radicals and prevents lipid peroxidation in biological systems.

As listed in Table 3.1, the FRS rate constants, k_S, of ubiquinol-10 and α-tocopherol are similar in ethanol. On the other hand, the rate constants of α-tocopherol in diethyl ether, benzene, and *n*-hexane are 3.7 to 9.2 times larger than the corresponding rate constants for ubiquinol-10. However, the difference in the rate constants is less than one order of magnitude. The result suggests that both α-tocopherol and ubiquinol-10 may relate to the scavenging of the active oxygen radicals in biomembrane systems.

3.3.2 Kinetic Study of Peroxyl-Radical-Scavenging Action of Biological Hydroquinones in Solution

Efficient phenolic antioxidants such as α-tocopherol are well known to terminate free radical chain peroxidations by trapping two peroxyl radicals according to Eqs. 3.1 and 3.9, so that the so-called stoichiometric factor, *n*, is 2.[3,18]

$$\mathrm{LOO\bullet} + \mathrm{TocH} \xrightarrow{k_{inh}} \mathrm{LOOH} + \mathrm{Toc\bullet} \tag{3.1}$$

$$\mathrm{LOO\bullet} + \mathrm{Toc\bullet} \xrightarrow{\text{fast}} \text{nonradical combination products} \tag{3.9}$$

The expression for suppressed oxygen uptake during the inhibition period is given by Eq. (3.10). In calculations, we used the reported value[3,18] of 41 $M^{-1}s^{-1}$ for the propagation rate constant, k_p, for peroxidation of styrene at 30°C.

$$-d[\mathrm{O_2}]/dt = (k_P/k_{inh} \times [\mathrm{LH}]\, R_i)/n\,[\mathrm{TocH}] \tag{3.10}$$

The rate of radical chain initiation, R_i, is known for initiation by the azo-initiator AIBN (2,2'-azobisisobutyronitrile), and can be measured by the induction period method using Eq. (3.11), where τ is the length of the induction period during suppressed oxygen uptake,

$$R_i = n[\mathrm{TocH}]/\tau \tag{3.11}$$

$$\Delta[\mathrm{O_2}]_t = -(k_P/k_{inh}) \times [\mathrm{LH}] \ln(1 - t/\tau) \tag{3.12}$$

TABLE 3.2
Second-Order Rate Constants (k_{inh}) for the Reaction of Peroxyl Radical (LOO•) with Biological Hydroquinones and 2,3,5-trimethyl-4-methoxyphenol (TMMP) in Styrene Solution at 30 °C, Thermally Initiated by AIBN

Biological Hydroquinones	$10^{-6}\,k_{inh}$ ($M^{-1}\,s^{-1}$)	*n*
$UQ_{10}H_2$ **1**	0.35	1.0
UQ_0H_2 **2**	0.31	1.9
α-TQH_2 **5**	1.5	>0.50
β-TQH_2 **6**	1.1	0.64
γ-TQH_2 **7**	0.80	>0.50
$TMQH_2$ **8**	1.4	>0.50
TMMP	1.3	2.0
α-TocH	2.9	2.0[a]

[a] See Ref. 3.

The antioxidant activity, represented by the absolute rate constant for inhibition, k_{inh}, is determined by measuring the oxygen uptake during the course of the inhibition period. For calculations, the integrated form of the inhibition period, Eq. 3.12, was used as before.[32] The rate constants were obtained from the slopes of the plots of $\Delta[O_2]_t$ versus $-\ln(1 - t/\tau)$, which equal $k_p[LH]/k_{inh}$. The k_{inh} values obtained for biological hydroquinones are listed in Table 3.2.[32]

As listed in Table 3.2, the hydroquinones typically gave relatively low stoichiometric factors. Therefore, we determined the k_{inh} of 2,3,6-trimethyl-4-methoxyphenol (TMMP)[32] to compare with the corresponding hydroquinone ($TMQH_2$ **8**). The latter gave about the same k_{inh} despite low *n* value. The antioxidant activities of the tocopherol hydroquinones (**5–7**) are all lower than the known values for the corresponding tocopherols (Table 3.2, and Refs. 3 and 18).

The ubiquinol-10 and -0 (**1** and **2**) have significantly lower antioxidant activities than the other hydroquinones studied (Table 3.2). These results are in general agreement with reports showing that the relative k_{inh} of ubiquinol-10 was 10 times less than α-tocopherol during peroxidation of methyl linoleate in *n*-hexane[8] and 0.34–0.39 times as reactive as α-tocopherol during autoxidation of egg phosphatidylcholine in organic solvents.[34] However, in the latter case, formation of reverse micelles complicated the system compared to homogeneous solutions.

The antioxidant activities of the ubiquinol-10 and -0 in solutions are of particular interest in view of their significant behavior as antioxidants in natural systems.[6–13,34,35] The lower k_{inh} values observed here account for the lower relative antioxidant activity reported earlier in solution.[8,34] The two adjacent methoxy groups appear to depress the antioxidant activity relative to the polyalkylhydroquinones **5**, **6**, **7**, and **8** (Table 3.2). We suggest that the two adjacent methoxy groups are forced out-of-plane so that their main effect is the inductive electron withdrawal by oxygen, and as a result, the developing phenoxyl radical is destabilized.

Remarkably different results have been reported for the antioxidant activity of ubiquinol-10 in model biological systems compared to that in solution, and these effects have been reviewed.[35] It appears to be as effective as α-tocopherol against peroxidative attack on liposomal membranes[7,8] but more efficient than α-tocopherol in protecting human low density lipoprotein (LDL).[9,35] Such differences in the action of ubiquinol have been interpreted in terms of different particle sizes of the microenvironment whereby in small LDL particles, the ubiquinol may function through the semiquinone radical $UQ_{10}H$• to "export radicals" (e.g., HOO•) from the small particle into the aqueous phase. This phenomenon is expected to be controlled by the volume of the environment

available to this antioxidant. We speculate that the antioxidant activity of ubiquinols depends on the system used and would vary accordingly as follows: LDL > liposomes > homogeneous solution.

The overall efficiency of an antioxidant is determined by the number of radicals trapped per molecule, the stoichiometric factor, *n*, as well as the inhibition rate constant (k_{inh}). Various factors may result in nonintegral values for *n*. For example, *n* factors greater than the "expected" 2 can arise for less active inhibitors if self-termination of the peroxyls is significant,[36] so that the inhibition period becomes longer than implied by Eqs. 3.1 and 3.9. Stoichiometric factors for most phenols are usually 2. In fact, the *n* factors for the α-tocopherol and TMMP (Table 3.2) are 2. On the other hand, values of *n* for derivatives of the hydroquinones were frequently found to even drop below 0.5, and in this case, large errors are involved in their determination. This is undoubtedly due to the ease of oxidation in solution of most of these hydroquinones.

3.4 VITAMIN E REGENERATION REACTION OF BIOLOGICAL HYDROQUINONES

3.4.1 Kinetic Study of the Regeneration Reaction of Tocopheroxyl Radical by Biological Hydroquinones in Solution

α-Tocopheroxyl shows absorption peaks with λ_{max} = 428 and 410 nm in isopropyl alcohol/water (5:1, v/v) mixtures (Figure 3.5). Upon addition of an isopropyl alcohol/water solution with excess γ-TQH_2 to an isopropyl alcohol/water solution with α-tocopheroxyl, the absorption spectrum of α-tocopheroxyl disappears immediately. Figure 3.5 shows an example of the interaction between α-tocopheroxyl and γ-TQH_2 **7** (0.372 mM) in an isopropyl alcohol/water (5:1, v/v) solution.

The oxidation rates of γ-TQH_2 **7** by α-tocopheroxyl were studied spectro-photometrically with a stopped-flow technique in the presence of excess γ-TQH_2 **7** in an isopropyl alcohol/water solution (Reaction 4). The details of the experiments are reported in a previous paper.[26]

$$\text{Toc}\bullet + \gamma\text{-TQH}_2 \xrightarrow{k_r} \text{TocH} + \gamma\text{-TQH}\bullet \tag{3.4}$$

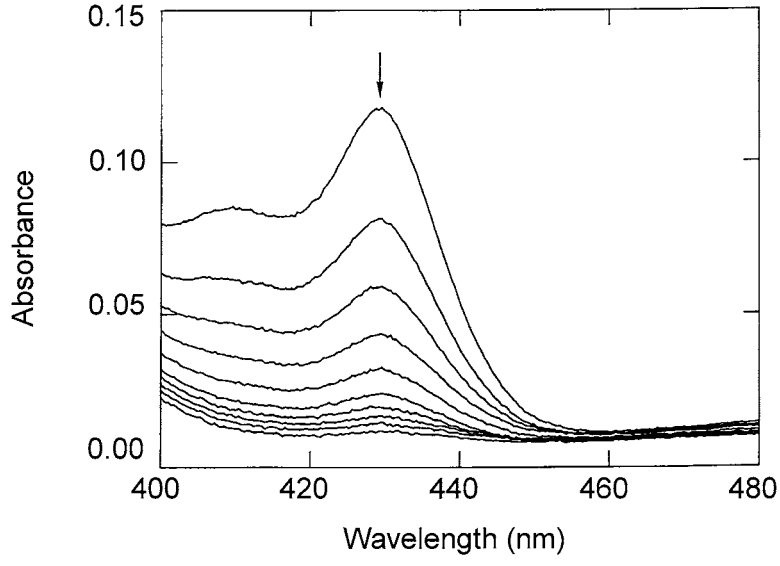


FIGURE 3.5 Change of electronic absorption spectrum of α-tocopheroxyl radical for reaction of α-tocopheroxyl with γ-TQH_2 **7** in isopropyl alcohol/water (5:1, v/v) mixtures at 25.0°C. $[\gamma\text{-TQH}_2]_{t=0} = 3.72 \times 10^{-4}$ M. The spectra were recorded at 4.5 ms intervals. The arrow indicates a decrease in absorbance with time.

TABLE 3.3
Second-Order Rate Constants (k_r) and Relative Rate Constants (k_r (HQ)/k_r ($UQ_{10}H_2$)) for the Reaction of α-tocopheroxyl and 5,7-diisopropyltocopheroxyl with Biological Hydroquinones 1–8 in Ethanol and Benzene Solutions at 25.0°C.

	α-Toc•				5,7-Di-*i*Pr- Toc•			
	Ethanol		Benzene		Ethanol		Benzene	
Biological Hydroquinones	k_s ($M^{-1}s^{-1}$)	k_r (HQ)/k_r($UQ_{10}H_2$)	k_r ($M^{-1}s^{-1}$)	k_r (HQ)/k_r($UQ_{10}H_2$)	k_r ($M^{-1}s^{-1}$)	k_r (HQ)/k_r($UQ_{10}H_2$)	k_r ($M^{-1}s^{-1}$)	k_r (HQ)/k_r($UQ_{10}H_2$)
($UQ_{10}H_2$) **1**	2.15×10^5	1.00	3.74×10^5	1.00	3.64×10^4	1.00	8.48×10^4	1.00
UQ_0H_2 **2**	8.08×10^4	0.38	3.30×10^5	0.88	1.28×10^4	0.35	9.23×10^4	1.09
VK_1H_2 **3**	$>10^6$ (7.7×10^6)[a]		$>10^6$		1.30×10^6	35.7	$>10^6$	
VK_3H_2 **4**	$>10^6$ (3.4×10^6)[a]		$>10^6$		5.81×10^5	16.0	$>10^6$	
α-TQH_2 **5**	5.49×10^5	2.55	$>10^6$		9.78×10^4	2.69	1.02×10^6	12.0
β-TQH_2 **6**	3.51×10^5	1.63	$>10^6$		5.78×10^4	1.59	6.10×10^5	7.19
γ-TQH_2 **7** (PQH_2 model)	3.40×10^5	1.58	$>10^6$		6.64×10^4	1.82	8.12×10^5	9.58
$TMQH_2$ **8**	2.85×10^5	1.33	$>10^6$		5.18×10^4	1.42	5.64×10^5	6.65

[a] Values estimated from the k_r (HQ)/k_r ($UQ_{10}H_2$) values for the reaction of 5,7-diisopropyltocopheroxyl with VK_1H_2 and VK_3H_2 in ethanol (see text).

The rate was measured by following the decrease in absorbance at 428 nm of the α-tocopheroxyl radical. The pseudo first-order rate constants (k_{obsd}) observed at 428 nm were linearly dependent on the concentration of γ-TQH_2, and thus the rate law is expressed as follows:

$$-d[\text{Toc}\bullet]/dt = k_{obsd}[\text{Toc}\bullet] = k_r[\gamma\text{-TQH}_2][\text{Toc}\bullet] \quad (3.13)$$

where k_r is the second-order rate constant for oxidation of γ-TQH_2 by the α-tocopheroxyl radical. The k_r values are obtained by plotting k_{obsd} against [γ-TQH_2]. Similar measurements were performed for the reaction of α-tocopheroxyl with biological hydroquinones **1–8** in ethanol, benzene, and isopropyl alcohol/water (5:1, v/v) solutions. The reaction rates for hydroquinones **1–8** with 5, 7-diisopropyltocopheroxyl in ethanol, benzene, and isopropyl alcohol/water solutions were also measured. The k_r values obtained are summarized in Tables 3.3 and 3.4.[15,26] The reaction rates for VK_1H_2 and VK_3H_2 with α-tocopheroxyl in ethanol, benzene, and isopropyl alcohol/water solutions were very fast, and we did not succeed in measuring the k_r values for these hydroquinones.

The kinetic study of the reaction between tocopheroxyl (vitamin E radical) and $UQ_{10}H_2$ was performed (Reaction 4).[15,26] For instance, the k_r values obtained for α-tocopheroxyl are 3.74×10^5 and 2.15×10^5 $M^{-1}s^{-1}$ in benzene and ethanol solutions at 25°C, respectively. The above k_r values were compared with those for vitamin C with α-tocopheroxyl reported by Packer et al.[37] ($k_r = 1.55 \times 10^6$ $M^{-1}s^{-1}$) and Scarpa et al.[38] ($k_r = 2 \times 10^5$ $M^{-1}s^{-1}$). The reaction is well known as an usual regeneration reaction of tocopheroxyl in biomembrane systems. The results indicated that ubiquinol-10 also regenerates the tocopheroxyl to tocopherol and prevents lipid peroxidation in various tissues and mitochondria.

Further, the reaction rates of seven kinds of biologically important hydroquinones, including vitamin K_1 HQ and α-tocopherol-HQ, with tocopheroxyl were measured in ethanol and benzene solutions.[26] As listed in Table 3.3, hydroquinones **3–8**, (not UQ_0H_2 **2**) showed rate constants larger than that of $UQ_{10}H_2$ **1** in ethanol and benzene solutions. In particular, the rates of the regeneration reaction of α-tocopherol with VK_1H_2 and VK_3H_2 in ethanol and benzene solutions were too fast to determine, and only the minimum value of the rate constant could be estimated (10^6 $M^{-1}s^{-1}$). On the other hand, the rates of the regeneration reaction (k_r) of 5,7-diisopropyltocopherol with VK_1H_2 and VK_3H_2 in ethanol were determined and found to be 36 and 16 times larger than that of $UQ_{l0}H_2$, respectively. As is clear from the k_r values listed in Table 3.3, the rate of the regeneration reaction of tocopherol with the above biological HQs increases in the order of $UQ_0H_2 < Q_{10}H_2 < \beta$-$TQH_2 \sim \gamma$-$TQH_2 \sim$

TABLE 3.4
Second-Order Rate Constants (k_r) and Relative Rate Constants (k_r (HQ)/k_r ($UQ_{10}H_2$)) for the Reaction of α-tocopheroxyl and 5,7-diisopropyltocopheroxyl with Biological Hydroquinones 1–8 in Isopropyl Alcohol/Water (5:1, v/v) Solutions at 25.0 °C and Peak Oxidation Potentials (E_P) for Hydroquinones 1–8

	α-Toc•		5,7-Di-*i*Pr-Toc•		
Biological Hydroquinones	k_r ($M^{-1}s^{-1}$)	k_r (HQ)/k_r ($UQ_{10}H_2$)	k_r ($M^{-1}s^{-1}$)	k_r (HQ)/k_r ($UQ_{10}H_2$)	E_P (mV vs. SCE[a])
$UQ_{10}H_2$ **1**	2.54×10^5	1.00	5.33×10^4	1.00	930
UQ_0H_2 **2**	8.80×10^4	0.35	1.52×10^4	0.29	960
VK_1H_2 **3**	$>10^6$ (1.3×10^7)[b]		2.69×10^6	50.5	
VK_3H_2 **4**	$>10^6$ (4.0×10^6)[b]		8.35×10^5	15.7	770
α-TQH_2 **5**	8.15×10^5	3.21	1.53×10^5	2.87	830
β-TQH_2 **6**	4.38×10^5	1.72	7.98×10^4	1.50	870
γ-TQH_2 **7**	4.10×10^5	1.61	1.05×10^5	1.97	880
$TMQH_2$ **8**	4.12×10^5	1.62	6.72×10^4	1.26	870
Sodium ascorbate	2.68×10^6	10.6	6.32×10^4	1.19	
Sodium ascorbate	1.55×10^6 [c]	6.10			
Sodium ascorbate	2×10^5 [d]	0.8			

[a] Saturated calomel electrode.
[b] Values estimated from the k_r (HQ)/k_r ($UQ_{10}H_2$) values for the reaction of 5,7-diisopropyltocopheroxyl with VK_1H_2 and VK_3H_2 in isopropyl alcohol/water (5:1, v/v) mixtures (see text).
[c] The k_r value obtained in water/isopropyl alcohol/acetone (50:40:10, v/v) mixtures (Ref. 37).
[d] The k_r value obtained in liposome systems (Ref. 38).

$TMQH_2 < \alpha$-$TQH_2 < VK_3H_2 < VK_1H_2$ regardless of the polarity of the solvent, i.e., the polarity of the reaction field. The results suggest that all the biological hydroquinones studied in this work also regenerate the tocopheroxyl to tocopherol and prevent lipid peroxidation in biological systems.

As described above, the k_r values for the reaction between tocopheroxyl and HQs were measured in an ethanol solution. As listed in Table 3.3, for each HQ, the absolute k_r value obtained for α-tocopheroxyl is ~5.8 ± 0.6 times larger than that for 5,7-diisopropyltocopheroxyl. On the other hand, the relative rates (HQs 1:2:5:6:7:8 = 1.00:0.38:2.55:1.63:1.58:1.33) obtained for α-tocopheroxyl are in good agreement with those (1.00:0.35:2.69:1.59:1.82:1.42) obtained for 5,7-diisopropyltocopheroxyl. The results suggest that the relative reactivities of HQs in solution do not depend on the kinds of tocopheroxyl radicals used even though the absolute rates are different. Therefore, the k_r values for the reaction of α-tocopheroxyl with VK_1H_2 and VK_3H_2 in ethanol were estimated to be (7.7 ± 0.8) × 10^6 and (3.4 ± 0.4) × 10^6 $M^{-1}s^{-1}$ from the relative rate of constants (k_r (HQ)/k_r ($UQ_{10}H_2$)) obtained for the reaction of 5,7-diisopropyltocopheroxyl with VK_1H_2 and VK_3H_2, respectively. Similarly, the values k_r for the regeneration reaction of α-tocopherol with VK_1H_2 and VK_3H_2 in isopropyl alcohol/water (5:1, v/v) mixtures were estimated to be (1.3 ± 0.2) × 10^7 and (4.0 ± 0.4) × 10^6 $M^{-1}s^{-1}$ from the k_r (HQ)/k_r ($UQ_{10}H_2$) values for 5,7-diisopropyltocopheroxyl (see Table 3.4), respectively. These rate constants are very fast, and thus we can expect high antioxidant action for VK_1H_2 and VK_3H_2 compounds in biological systems.

3.4.2 Comparison between Rates of Vitamin E Regeneration Reaction with Biological Hydroquinones and Vitamin C in Solution

Ascorbic acid is a dibasic acid and thus, in an aqueous solution system, can exist in three different molecular forms [L-ascorbic acid (AsH_2), ascorbate monoanion (ASH^-), and dehydroascorbate dianion (As^{2-})] depending on the pH value (Figure 3.2). In a previous paper, a kinetic study of the reaction between vitamin C (*L*-ascorbic acid) and 7-*tert*-butyl-5-isopropyltocopheroxyl in a Triton X-100 micellar solution was performed using stopped-flow spectrophotometry.[39] The second-order rate constants (k_r) obtained showed notable pH dependence with a broad maximum around pH 8. A good correlation between the rate constants and the mole fraction of ascorbate monoanion (AsH^-) was observed, showing that ascorbate (AsH^-) can regenerate the tocopherol from tocopheroxyl in biological systems. Therefore, we measured the reaction rates for sodium ascorbate (Na^+AsH^-) with the tocopheroxyl radical in isopropyl alcohol/water (5:1, v/v) mixtures because Na^+AsH^- is insoluble in ethanol and benzene solutions. The k_r values obtained for biological hydroquinones **1–8** were compared with that for vitamin C (sodium ascorbate) with α-tocopheroxyl (see Table 3.4). The k_r value obtained for the reaction between Na^+AsH^- and α-tocopheroxyl is 2.68×10^6 $M^{-1}s^{-1}$. This value is 2 times larger than that (1.55×10^6 $M^{-1}s^{-1}$) reported by Packer et al.[37] for the same reaction in water/isopropyl alcohol/acetone (50:40:10, v/v) mixtures using the pulse radiolysis method.

For instance, comparing the k_r value (2.68×10^6 $M^{-1}s^{-1}$) obtained for the reaction of α-tocopheroxyl with Na^+AsH^- (Reaction 2) with those (2.54×10^5 and 8.15×10^5 $M^{-1}s^{-1}$) obtained for the reaction of α-tocopheroxyl with $UQ_{l0}H_2$ and α-TQH_2 (Reaction 4) in isopropyl alcohol/water mixtures, the former is ~11 and 3 times as reactive as the latter, respectively. On the other hand, the k_r value obtained for Na^+AsH^- is 4.9 and 1.5 times smaller than those obtained for VK_1H_2 and VK_3H_2, respectively. Therefore, the order of magnitude of k_r values obtained for HQs and Na^+AsH^- is $VK_1H_2 > VK_3H_2 > AsH^- > \alpha$-$TQH_2 > \beta$-$TQH_2 \sim \gamma$-$TQH_2 \sim TMQH_2 > UQ_{10}H_2 > UQ_0H_2$ in isopropyl alcohol/water (5:1, v/v) mixtures. The role of vitamin E (α-, β-, γ- and δ-tocopherols) as an important biological antioxidant has been well recognized in recent years.[1,2] The antioxidant properties of the tocopherols have been ascribed to the initial oxidation by a peroxyl radical of the phenolic hydroxyl group, producing a tocopheroxyl radical (Reaction 1).[3] As reported previously, absolute reactivities (k_{inh}) of tocopherols to LOO• vary depending on the number of alkyl substituents,[17,33] i.e., the antioxidant activity of these tocopherol compounds increases as the total electron-donating capacity of the alkyl substituents on the aromatic ring increases. The log of the second-order rate constants (k_{inh}) obtained for α-, β-, γ- and δ-tocopherols was found to correlate with their E_P values.[17]

As described above, the rate of the regeneration reaction of α-tocopherol with biological hydroquinones **1–8** has been measured in isopropyl alcohol/water (5:1, v/v) mixtures. Furthermore, the E_P values for these HQs were also measured. Consequently, the values of log k_r for HQs have been plotted against E_P. As shown in Figure 3.6, a plot of log k_r versus E_P is linear over most of the range, with a slope of -6.7 V^{-1} (correlation coefficient = 0.97). The same correlation is given for the reaction of HQs with the 5,7-diisopropyltocopheroxyl radical, showing a slope of -8.1 V^{-1} (correlation coefficient = 0.96) (Figure 3.6).

In fact, as listed in Tables 3.3 and 3.4, the k_r and E_P values for β-TQH_2, γ-TQH_2, and $TMQH_2$ are similar to each other in solution because these HQs have three alkyl substituents on the aromatic ring. α-TQH_2 with four alkyl substituents showed larger k_r and smaller E_P values than β-TQH_2, α-TQH_2, and $TMQH_2$. Plastoquinone also has three alkyl substituents on the aromatic ring, and we can expect that the rate of the regeneration reaction with PQH_2 will be similar to those with β-TQH_2, γ-TQH_2, and $TMQH_2$ in solution. In particular, both PQH_2 and γ-TQH_2 have two methyl substituents at positions 2 and 3 and a long-alkyl-chain at position 6, and thus the activities of PQH_2 and γ-TQH_2 are thought to be similar. The results suggest that PQH_2 also regenerates the tocopheroxyl to tocopherol and prevents lipid peroxidation in biological systems.

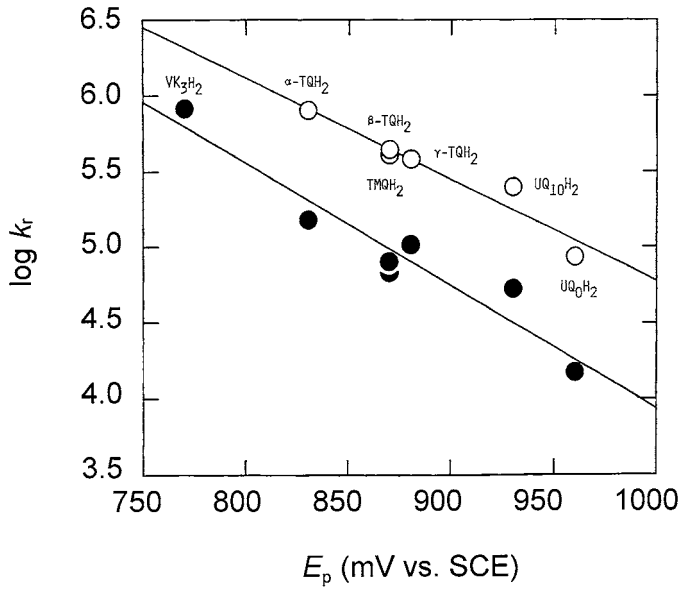


FIGURE 3.6 Plots of log k_r for reaction of biological hydroquinones **1–8** with α-tocopheroxyl and 5,7-diisopropyltocopheroxyl vs. E_P for hydroquinones **1–8**. The plots for α-tocopheroxyl (O) and 5,7-diisopropyltocopheroxyl (•) gave linear fits with slopes of -6.7 and -8.1 V^{-1} and correlation coefficients of 0.97 and 0.96, respectively. SCE, saturated calomel electrode.

If the biological HQs coexist with α-tocopherol in membrane or tissues, the rate of the disappearance of LOO• is represented by Eq. 3.14.

$$-d\,[\text{LOO}\bullet]/dt = k_{\text{inh}}^{(3)}\,[\text{HQ}]\,[\text{LOO}\bullet] + k_{\text{inh}}^{(1)}\,[\alpha\text{-TocH}][\text{LOO}\bullet] \tag{3.14}$$

The peroxyl-radical-scavenging Reaction 3 of HQ may compete with Reaction 1 of α-tocopherol. The rate of scavenging of the peroxyl radical depends on the product of the second-order rate constant and the concentration of each molecule, as shown in Eq. 3.14. Similarly, if the biological HQs coexist with vitamin C (ascorbate (AsH^-)) in biological systems, the rate of regeneration of Toc• is represented by Eq. 3.15.

$$-d\,[\text{Toc}\bullet]/dt = k_r^{(4)}\,[\text{HQ}]\,[\text{Toc}\bullet] + k_r^{(2)}\,[\text{AsH}^-]\,[\text{Toc}\bullet] \tag{3.15}$$

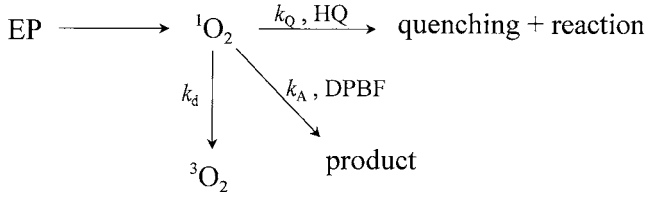
Naumov and Khrapova[14] and Mukai, et al.[32] measured the second-order rate constants (k_{inh}) for the reaction of the peroxyl radical with ubiquinol-9 in ethylbenzene and ubiquinol-10 in chlorobenzene using the chemiluminescence and O_2 consumption methods, respectively. The k_{inh} values obtained for ubiquinol-9 (3.4×10^5 $M^{-1}s^{-1}$) and ubiquinol-10 (3.5×10^5 $M^{-1}s^{-1}$, Table 3.2) are only an order of magnitude lower than the k_{inh} value (3.3×10^6 $M^{-1}s^{-1}$) for α-tocopherol in ethylbenzene.[40] The molar ratio of ubiquinone-10 plus ubiquinol-10 to α-tocopherol in the inner mitochondrial membrane is ~10:1,[13] which is closer to or lower than the 1:1 ratio in other biomembranes,[10] and ~1:20 in lipoproteins.[7,9] Furthermore, ubiquinol exists in relatively high concentrations in various tissues and mitochondria,[41–43] and the percentages of ubiquinols to total ubiquinones are 41.6, 32.4, and 45.2% in guinea pig heart, rat heart, and guinea pig heart mitochondrial fractions, respectively.[43] So we can expect that ubiquinol-10 contributes to the scavenging of the peroxyl radical, at least in the former two systems.

We studied the regeneration reaction of α-tocopherol with $UQ_{10}H_2$ using stopped-flow spectrophotometry.[15] The k_r values obtained for the reaction between $UQ_{l0}H_2$ and α-tocopheroxyl are 3.74×10^5 and 2.15×10^5 $M^{-1}s^{-1}$ in benzene and ethanol solutions at 25.0°C, respectively. The above k_r values were compared with those for vitamin C with α-tocopheroxyl reported by Packer et al.[37] ($k_r = 1.55 \times 10^6$ $M^{-1}s^{-1}$) and Scarpa et al.[38] ($k_r = 2 \times 10^5$ $M^{-1}s^{-1}$). The reaction of vitamin C with α-tocopheroxyl is well known as an usual regeneration reaction of tocopheroxyl in biomembrane systems. The results suggest that both Reactions 2 and 4 may contribute to the regeneration reaction of α-tocopherol.

The mitochondrial membrane is rich in unsaturated lipids and it is always in danger of oxidation. Consequently, mitochondrial membrane must be protected against peroxidation, and α-tocopherol appears to play an important role in this protection. The results of our kinetic study show that the ubiquinol-10 also strongly inhibits lipid peroxidation in mitochondria and other biomembranes. It may act as an antioxidant, first, by scavenging the active oxygen free radicals, such as LOO•, LO•, and HO• and, secondly, by regenerating the tocopherol. In fact, Frei et al.[7] and Yamamoto et al.[8] investigated the antioxidant activity of ubiquinol in liposomal membranes. They reported that (i) ubiquinol is about as effective as α-tocopherol in inhibiting lipid peroxidation, and (ii) ubiquinol spares α-tocopherol when both antioxidants are present in the same liposomal membranes. Stocker et al.[9] reported that ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than α-tocopherol. On the other hand, Kagan et al.[10,11] studied the antioxidant activity of ubiquinol in microsomes and mitochondria. They reported that (i) direct radical scavenging effects of ubiquinols may be negligible in the presence of comparable or higher concentrations of tocopherols, and (ii) antioxidant effects of ubiquinols are due to their ability to recycle tocopherols from tocopheroxyls. The reason is not clear at present why direct radical scavenging effects of ubiquinols are negligible in microsomes and mitochondria and are about as effective as α-tocopherol in liposomal systems. Concentration of biological hydroquinones such as VK_1H_2, PQH_2, and α-TQH_2 in biological systems has not been reported as far as we know. However, the result of the present kinetic study strongly suggests that these HQs also function as antioxidants in biological systems such as mitochondria, other biomembranes, and various tissues.

3.5 QUENCHING REACTION OF SINGLET OXYGEN BY BIOLOGICAL HYDROQUINONES

Singlet oxygen ($^1O_2$) was generated by the thermal decomposition of the 3-(1,4-epidioxy-4-methyl-l, 4-dihydro-l-naphthyl)propionic acid (EP) (Figure 3.2).[28] 2,5-Diphenyl-3,4-benzofuran (DPBF) was used as standard compound. The overall rate constants k_Q ($= k_q + k_{cr}$) for the reaction of $^1O_2$ with hydroquinones **1**, **2**, **5–13** were determined in ethanol by Eq. 3.16 derived from the steady-state treatment of Scheme 1.[44]



$$S_O/S_S = 1 + [(k_q + k_{cr})/k_d][HQ] \tag{3.16}$$

where S_O and S_S are slopes of the first-order plots of disappearance of $^1O_2$ acceptor, DPBF, in the absence and presence of hydroquinone, respectively; k_d is the rate of deactivation of $^1O_2$ in ethanol. Solutions containing EP (3.8×10^{-3} M), DPBF (3.9×10^{-5} M) and various amounts of

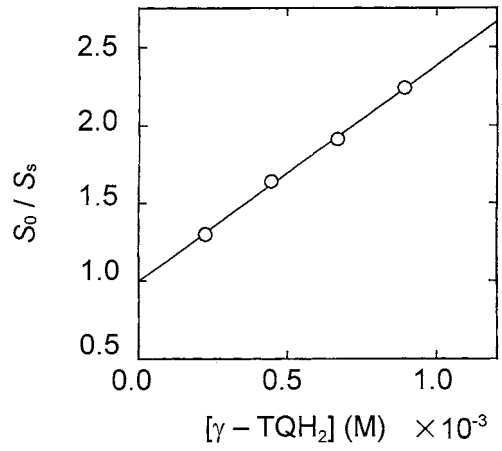


FIGURE 3.7 Plot of S_O/S_S vs. concentration of γ-tocopherol-hydroquinone.

hydroquinone (0–2 mM) in ethanol were reacted at 35 °C. The disappearance of DPBF was measured at 411 nm. The details of experiments are reported in previous papers.[28] S_O/S_S vs. [γ-TQH_2] plots for γ-tocopherol-hydroquinone **7** are shown in Figure 3.7. The overall rate constants (k_Q) were calculated by using the value of k_d in ethanol ($k_d = 8.3 \times 10^4\ s^{-1}$), reported by Merkel and Kearns.[45] Similarly, hydroquinone derivatives **1**, **2**, **5–13** were reacted with the $^1O_2$ in ethanol. The k_Q values obtained were summarized in Table 3.5, together with those reported for α-, β-, γ-, and δ-tocopherols.[24] The experimental error in k_Q value for each tocopherol was ±8% at maximum.

As listed in Table 3.5, hydroquinone (QH_2) (**13**), 2-methylhydroquinone (2-MQH_2) (**12**), 2, 3-dimethylhydroquinone (2,3-$DMQH_2$) (**9**) and 2,3,5-trimethylhydroquinone ($TMQH_2$) (**8**) are about 6.8%, 21%, 41%, and 56% as reactive as α-tocopherol, respectively. The result indicates that the rate of quenching of $^1O_2$ by these hydroquinone derivatives varies depending on the number of methyl substitutions.[24] In fact, β-TQH_2 and γ-TQH_2, having three alkyl substituents, have rate constants similar to that of $TMQH_2$ (**8**). The result indicates that the rate constants increase as the total electron-donating capacity of the alkyl substituents in the aromatic ring increases. Further, plastoquinone, which is very important as an electron carrier in photosynthetic systems, also has three alkyl substituents on the aromatic ring, and we can expect that the rate constant k_Q of plastoquinol (PQH_2) (Figure 3.1) will be similar to those of β-TQH_2, γ-TQH_2 and $TMQH_2$ in solution.

Measurements of peak oxidation potential, E_P, of biological HQs (**1**, **2**, and **5–7**) and related HQs (**8–13**) have been reported in previous papers.[26,27] These values are listed in Table 3.5. The values of log k_Q for HQs (**1**, **2**, and **5–13**) have been plotted against E_P. In fact, as shown in Figure 3.8, log k_Q correlates roughly with E_P with a slope of $-3.6\ V^{-1}$ (correlation coefficient = −0.87).[24,27,46] The HQs that have smaller E_P values show higher reactivities. The result suggests that the transition state in the above $^1O_2$ quenching reaction by HQs has the property of a charge-transfer intermediate.

As reported in a previous paper, the free-radical-scavenging rate constants, k_S, of $UQ_{10}H_2$ and α-tocopherol are similar in ethanol.[30] On the other hand, the rate constants of α-TQH_2, β-TQH_2, and γ-TQH_2 (PQH_2 model) are 1.6–2.9 times larger than that of α-tocopherol. The result suggests that α-tocopherol and these biological HQs may relate to the scavenging of the active oxygen radicals in biomembrane systems.

As listed in Table 3.5, the rate constants (k_Q) of quenching of $^1O_2$ by $UQ_{10}H_2$, UQ_0H_2, α-TQH_2, β-TQH_2, and γ-TQH_2 (PQH_2 model) are 23 ~ 55% smaller than that by α-tocopherol. However, the difference in the rate constants is less than one order of magnitude. Further, the rate constants of these biological HQs **1**, **2**, and **5–7** are similar to that of γ-tocopherol. VK_1H_2 and VK_3H_2 showed

TABLE 3.5
Second-Order Rate Constants (k_Q) and Relative Rate Constants (k_Q (HQ)/k_Q (α-TocH)) for the Reaction of Singlet Oxygen ($^1O_2$) with Hydroquinones 1,2,5-13 in Ethanol Solution at 35°C, and Peak Oxidation Potentials (E_P) for hydroquinones

Hydroquinones	10^{-8} k_Q (M^{-1} s^{-1})	k_Q (HQ) / k_Q (α-Toc)	E_P (mV vs. SCE[a])
$UQ_{10}H_2$ **1**	1.58	0.77	930
UQ_0H_2 **2**	0.93	0.45	960
α-TQH_2 **5**	1.26	0.61	830
β-TQH_2 **6**	1.22	0.59	870
γ-TQH_2 **7**	1.17	0.57	880
$TMQH_2$ **8** (PQH_2 model)	1.15	0.56	870
2,3-$DMQH_2$ **9**	0.85	0.41	930
2,5-$DMQH_2$ **10**	0.99	0.48	930
2,6-$DMQH_2$ **11**	0.54	0.26	930
2-MQH_2 **12**	0.44	0.21	1000
QH_2 **13**	0.14	0.068	1090
α-Tocopherol	2.06[b]	1.00	860[b]
β-Tocopherol	1.53[b]	0.74	920[b]
γ-Tocopherol	1.38[b]	0.67	930[b]
δ-Tocopherol	0.53[b]	0.26	990[b]

[a] Saturated calomel electrode.
[b] See Ref. 24.

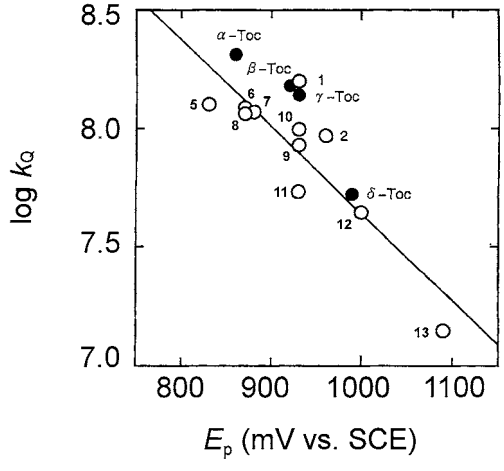


FIGURE 3.8 Plot of log k_Q vs. E_P for hydroquinones **1**, **2**, **5**–**13** (O) and α-, β-, γ-, and δ-tocopherols (•).

high activity in scavenging free radicals.[30] The rate constants k_S of VK_1H_2 and VK_3H_2 were 31 and 21 times larger than that of α-tocopherol, respectively. Thus, we can expect a large value of k_Q for both the VK_1H_2 and VK_3H_2.[24] However, VK_1H_2 and VK_3H_2 are unstable and oxidized easily in ethanol, and we did not succeed in determining the k_Q value.

Krashovsky et al.[47] reported the rate (k_Q) of quenching of $^1O_2$ by saturated and unsaturated fatty acids and lipids. The result indicates that the quenching rate increases as the number of double

bonds in fatty acid molecules increases. For instance, the values of rate constants observed are 9.0×10^3 $M^{-1}s^{-1}$ for stearic acid, 1.7×10^4 $M^{-1}s^{-1}$ for oleic acid, 4.2×10^4 $M^{-1}s^{-1}$ for linoleic acid, and 6.0×10^4 $M^{-1}s^{-1}$ for egg yolk phosphatidylcholine. The k_Q values ($(0.93 \sim 1.58) \times 10^8$ $M^{-1}s^{-1}$) observed for biological HQs **1**, **2**, and **5–7** are 3 to 4 orders of magnitude larger than those for fatty acids and phospholipids. The result suggests that these biological HQs may relate to the quenching of the singlet oxygen and prevent lipid peroxidation in biological membranes.

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Section 1B

Biochemical Mechanisms in Electron Transport in Mitochondria and Extramitochondrial Locations

4 Coenzyme Q Oxidation Reduction Reactions in Mitochondrial Electron Transport

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CONTENTS

4.1 EARLY CHEMIOSMOTIC RESPIRATORY SCHEMES

Coenzyme Q (ubiquinone-10), was discovered soon after plastoquinone, and structurally characterized at the end of the 1950s. Almost immediately, by 1961,[1] these closely related quinone compounds were included as components in the earliest formulations of Mitchell's chemiosmotic theory of energy conversion in prokaryotic and eukaryotic respiration and photosynthesis. Over the next 15 years, this theory would become accepted as the way by which all biological energy processing machinery converts the oxidation-reduction (redox) potential free energy of electron transfer systems into chemical potential energy stored by phosphorylation of ADP to form ATP. The key to the theory was the long sought identity of the energetic link between the dramatically dissimilar chemistries of oxidation-reduction and electron transfer on the one hand, and of ADP

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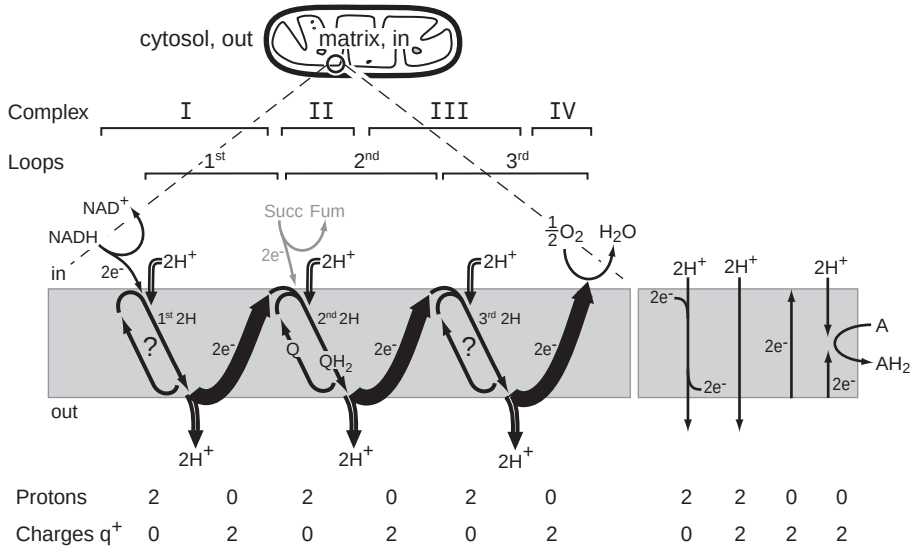

FIGURE 4.1 Early version of Peter Mitchell's chemiosmotic theory of energy conversion in the mitochondrial respiratory chain. The scheme emphasizes the simple linear sequence of alternating hydrogen-carrying and electrogenic electron transfer arms from NADH or succinate to dioxygen. Ubiquinone was suggested as responsible for the action of the second hydrogen-carrying (2H) arm; there were no viable candidates for the first and third 2H arms. On the right is a reminder of how transmembrane transfer of electrons and protons may be electrogenic or neutral.

phosphorylation and ATP hydrolysis on the other. An early version of Mitchell's chemiosmotic theory applied to a mitochondrial respiration is shown in Figure 4.1. The scheme serves well to remind us of the basic simplicity and elegance of Mitchell's theory in suggesting how the flow of electrons from NADH or succinate to dioxygen in a respiratory chain is organized to generate a delocalized transmembrane electrochemical gradient of protons ($\Delta\mu_{H+}$). Indeed, $\Delta\mu_{H+}$ proved to be the common energetic link, not only between respiration and the phosphorylation of ADP in the ATPase, but also between a myriad of other transport activities and chemistry in cells and their organelles; these are now well described in textbooks.

Figure 4.1 has been modified so that it forms a simple starting point for this chapter, which, in a step-by-step way, will outline the development of the roles played by ubiquinone in respiration. The respiratory scheme of Figure 4.1 indicates the small but essential part attributed to ubiquinone in early respiratory chains. Since that time, investigations have steadily uncovered new roles for ubiquinone, as well as seen its strategic position assume increasing importance in the energetic economy of respiratory and photosynthetic systems. Ubiquinone, as we shall describe here, has become recognized as a dominant and critical mechanistic player in three of the four major electron transfer complexes, Complexes I, II, and III of the mitochondrial respiratory chain.

Mitchell laid down similar electron and proton transfer schemes for a wide range of nonmitochondrial systems. Over 40 years, these systems developed in parallel to offer us a rich palette of information pertinent to both ubiquinone and the mitochondrion. Plant plastoquinone and phylloquinone have risen to the same dominating importance, holding essential mechanistic positions in all three major complexes of green plant chloroplasts, photosystems I and II and cytochrome (cyt) $b_6 f$ complex. It has become increasingly apparent that mechanisms involving quinones in membrane redox complexes of a wide variety of organisms belong to a single family; hence information from a variety of sources is relevant to events in mitochondria. This is important because very often other organisms offer experimental advantages and opportunities to look deeper into certain aspects of ubiquinone redox character and mechanism than is currently possible in mitochondrial studies.

These resources not only augment mechanistic mitochondrial studies, they also strengthen the foundations on which investigations can better address the seriously challenging issues of how the biochemistry of ubiquinone in the mitochondrion, with its unusual genetic makeup and its complex metabolic and energetic machinery, can affect human health.

4.2 UBIQUINONE MEMBRANE DIFFUSION CIRCUITS: FROM A SIMPLE TRANSMEMBRANE HYDROGEN-CARRYING ROLE IN ONE LOOP TO FORMULATIONS THAT ACCOUNT FOR HYDROGEN TRANSLOCATIONS IN ALL THREE LOOPS

The scheme in Figure 4.1 shows Mitchell's proposed sequence of three neutral hydrogen-carrying arms that alternate with charged electron transfer, electrogenic arms in his chemiosmotic respiratory chain. Mitchell called the combined neutral and electrogenic arms a "loop," and each loop in effect "pumped" one charged proton ion across the membrane for each electron traversing the loop. Each loop turned out to be roughly aligned with one of the membrane protein complexes that were steadily resolved and isolated with the help of metabolic studies and inhibitor action. Mitchell's early schemes always presented the transfer of electrons as pairs (2e-), perhaps for simplicity and accounting purposes; however, in describing the mechanistic aspects of more modern schemes, it is essential to acknowledge that electron transfer in respiratory chains, including associated catalytic sites, occur sequentially, one at a time.

For each NADH oxidized to NAD^+ on the matrix side of the mitochondrial inner membrane, two electrons (shown as 2e-) are introduced into the most reducing end (low redox potential) of the respiratory chain. By the time the two electrons passed through the three loops to reach dioxygen, Mitchell predicted that 6 protons and 6 charges would be translocated from the matrix side (in) to the cytosolic side (out) (i.e., 6 protons as chemical entities and 6 cationic charges, $6q^+$; see the conventions shown in the inset of Figure 4.1). Similarly, for each succinate oxidized to fumarate, two electrons also enter the chain from the matrix side after the first loop and then pass through two loops and translocate out a predicted 4 protons and $4q^+$. In each neutral arm, the "2H" moieties symbolize the combined two electrons and two protons transferred "bound together" as part of the integrated redox and acid/base chemistry proposed membrane diffusing redox carrier. When two electrons move with 2 protons, they are neutral overall, equivalent to "2H." It can be appreciated that this would contribute only to the generation of a concentration gradient of protons (ΔpH) across the membrane without building a transmembrane electric field. In contrast, the other arms were seen to carry charge and are called "electrogenic." In the electrogenic arms shown, two electrons are transferred (sequentially) from the outer to inner side of the membrane. This leaves, in effect, two positive charges on the outside. From our proton based convention (see Figure 4.1 right), this electrogenic electron transfer is electrically equivalent to $2q^+$ movement generating an electric potential across the membrane (positive on the outside relative to inside, $\Delta\psi$(out–in). The vectorial and functional complementary nature of the two arms of each loop combine to effectively carry a charged proton across the membrane and develop an electrochemical gradient of protons ($\Delta\mu_{H+}$) across the membrane. $\Delta\mu_{H+}$ can be approximated as follows:

$$\Delta\mu_{H+}(\text{out–in}) = \Delta\psi\,(\text{out–in}) - 0.06\,\Delta\text{pH}\,(\text{out–in}) \quad (\text{in V}) \tag{4.1}$$

Peter Mitchell recognized the unique properties of ubiquinone and plastoquinone as well as menaquinone in certain bacteria, assigning to them the role of diffusing carrier in the neutral 2H-carrying arm of the second loop to ubiquinone between Complexes I or II and III in mitochondria and bacteria. Analogous roles were assigned to plastoquinone in the light-driven electron transfer systems between PSII and the cyt b_6f complex in chloroplasts and to menaquinone in anaerobic bacteria. Three characteristics, evident from contemporary *in vitro* studies and biochemical analyses,

were critical to the proposed, central biological energetic role *in vivo* for ubiquinone:

1. Under physiological conditions in the membrane, reduction of ubiquinone (designated Q) to the reduced hydroquinone (designated QH_2) involves the exchange of two electrons and two protons and hence the molecule remains neutral, independent of whether it is in the Q or QH_2 form as shown in Eq. 4.2. Thus, the "2H" in the second loop typified the familiar and simplest description of the oxidation and reduction of QH_2 and Q.

$$Q + 2e^- + 2H^+ = QH_2 \quad (4.2)$$

2. The redox-independent neutral state, together with the highly hydrophobic character, kept the quinones clearly confined to the membrane interior of the cytoplasmic membranes. Their structure, comprising a long hydrocarbon tail and simple, relatively polar rings of the quinone head, was considered to be well suited to very strongly partition the ubiquinone into the membrane, as has since been quantitatively described in detail,[2,3] to permit relatively free diffusional mobility of the molecules in the bilayer part of the membrane, as well as to promote the headgroup to favor positions closer to the polar edges of the membrane-aqueous interface.
3. The quinones behaved like a rapidly equilibrating redox pool in molar excess of the respiratory and photosynthetic redox complexes in the same membrane. The diffusing pool character of the natural quinones not only connected the complexes together on a redox basis lateral to the membrane surface, but also contacted the membrane aqueous phases on each side of the membrane for proton exchange. Mitchell proposed that quinone was oxidized and reduced by different redox complexes at specific positions located on different sides of the membrane, to catalyze the translocation of the "2H" across the membrane coupled to oxidation and reduction. For example in the second loop, Q must be reduced on the mitochondrial matrix side (in) by electrons coming from NADH through the first loop (Complex I) or by electrons coming directly from succinate (Complex II). The location of the reduction on the matrix side promoted the binding of protons from the matrix side to form the neutral QH_2, which was then free to enter and diffuse in the membrane bilayer. The QH_2 was then viewed to be oxidized near the cytosolic side (out) of the membrane by an oxidant (cytochrome *c*, Complex III) and to release protons into the cytosol. This describes the role of ubiquinone in mitochondrial respiration as a redox pool linking Complexes I, II, and III on a redox basis and energetically serving as a simple 2H carrier at the second loop. This simple picture and modest role for ubiquinone is still generally correct, but as we shall see it has proven to be far from complete. Research demonstration and still-hypothetical models have revealed how ubiquinone can serve in the most elegant ways as the 2H carrier in all three loops.

While ubiquinone readily satisfied the role of the 2H-carrying arm of the second loop, the identity of diffusing 2H carriers for the first and third loops remained a serious problem for many years. By the mid-1970s, redox centers in respiratory complexes were being discovered and described at a rapid rate. These appeared to more than satisfy the electron transfer requirements of the electrogenic arms of the loops, but no other viable chemical candidates were discovered to equip the 2H carrying arms of the first and third loops. The solution to the third loop, associated with Complex III, was initiated in a proposal by Wikstrom and Berden in 1972[4] that was completed by Mitchell himself in 1976.[5] This gave rise to the "Q-cycle" scheme, which, over the past 25 years, has garnered considerable support and has been confirmed by many experiments. As shown in Figure 4.2, Mitchell's idea was that the ubiquinone also actually cycled in the membrane between two ubiquinone redox catalytic sites on the Complex III itself, which he called Q_o and Q_i sites for their location on the outer and inner side of the mitochondrial inner membrane. Thus, looking ahead to Figure 4.3A, the two sites were the terminals of the electron transferring electrogenic arm in the Complex III. This arm comprises a chain of two cytochromes *b*, named cyt b_L and b_H for their low and high

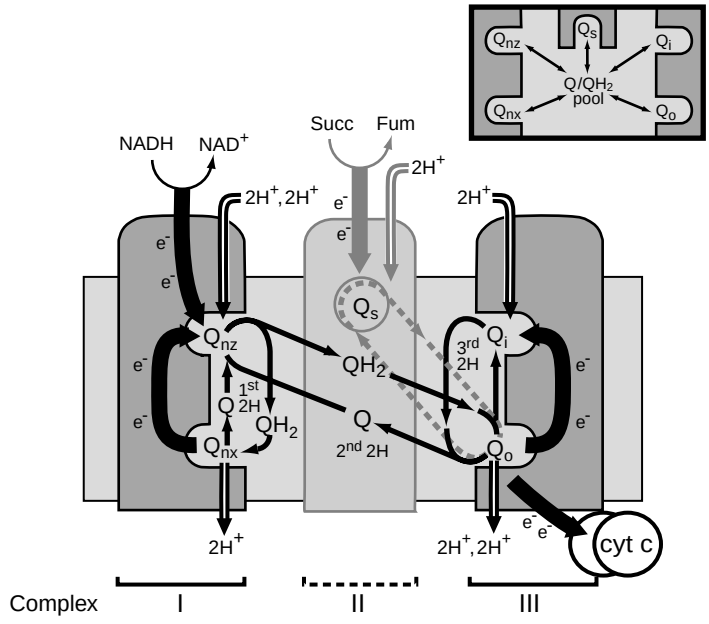

FIGURE 4.2 Modified scheme that suggests ubiquinone involvement in all three hydrogen-carrying arms. The extensive evidence supporting ubiquinone as the vehicle for the third 2H carrying arm associated with Complex III is applied as a hypothetical model for ubiquinone as the hydrogen carrier in the first 2H arm associated with Complex I. The action of Complex II is shown in gray to indicate its separate action regarding its interaction with the ubiquinone pool. The inset serves to indicate the fact that the different sites exchange with the Q_{pool} independently and the flow of Q and QH_2 are coupled in schemes for accounting purposes.

potentials, first resolved along with the cyt *c* and c_1[6–8] and the [2Fe2S] cluster[9] and characterized in the early 1970s in mitochondria and other organisms. The two hemes promote single electron tunneling directed across the membrane between the ubiquinone sites. Thus, in Complex III, the Q and the QH_2 cycle between the Q_o and the Q_i sites to form the 2H arm of the third loop of the respiratory chain.

Similar simultaneous attempts to explain the first loop associated with Complex I naturally drew on flavin[10] and the seven or eight iron sulfur clusters[11] discovered associated with the complex (see [12] for a discussion). However, these schemes have been rendered obsolete with the recent findings[13–18] that the flavin and iron sulfur cluster redox centers are not positioned in the membrane profile, but rather in a large section of Complex I subunits that extends as much as 100Å into the matrix aqueous phase[19] (see Figure 4.5). The iron-sulfur cluster identified at a position closest to the membrane aqueous interface on the matrix side has been identified as a [4Fe4S] cluster called N2. Thus, the only interfacial N2 and the membrane ubiquinone currently remain as candidate redox centers for the proton pumping mechanism at Complex I. In 1998, using just these components, we proposed a hypothetical model that accommodates ubiquinone as the 2H carrier of the first loop.[12] This suggested simply that ubiquinone also cycles around Complex I as shown in Figure 4.2 in a manner that shares symmetry with, and displays many of the characteristics of, the well-established "Q-cycle" scheme of Complex III, including two ubiquinone analogous to the Q_o and the Q_i sites; these were called Q_{nz} and Q_{nz} sites.

Despite these changes to the original scheme, as before, 6 protons and $4q^+$ ($2q^+$ with Complex I and $2q^+$ with Complex III) are translocated out of the mitochondrion for electron transfer from NADH to cyt *c*, and 4 protons and $2q^+$ ($2q^+$ with Complex III) are translocated out for electron transfer from succinate to cyt *c*. The remaining two charges (i.e., only $2q^+$) are associated with cyt *c* oxidase as in Figure 4.1. Although the main scheme of Figure 4.2 specifying the routes of the neutral arms of QH_2 and Q flow between the various reaction sites is useful for accounting purposes,

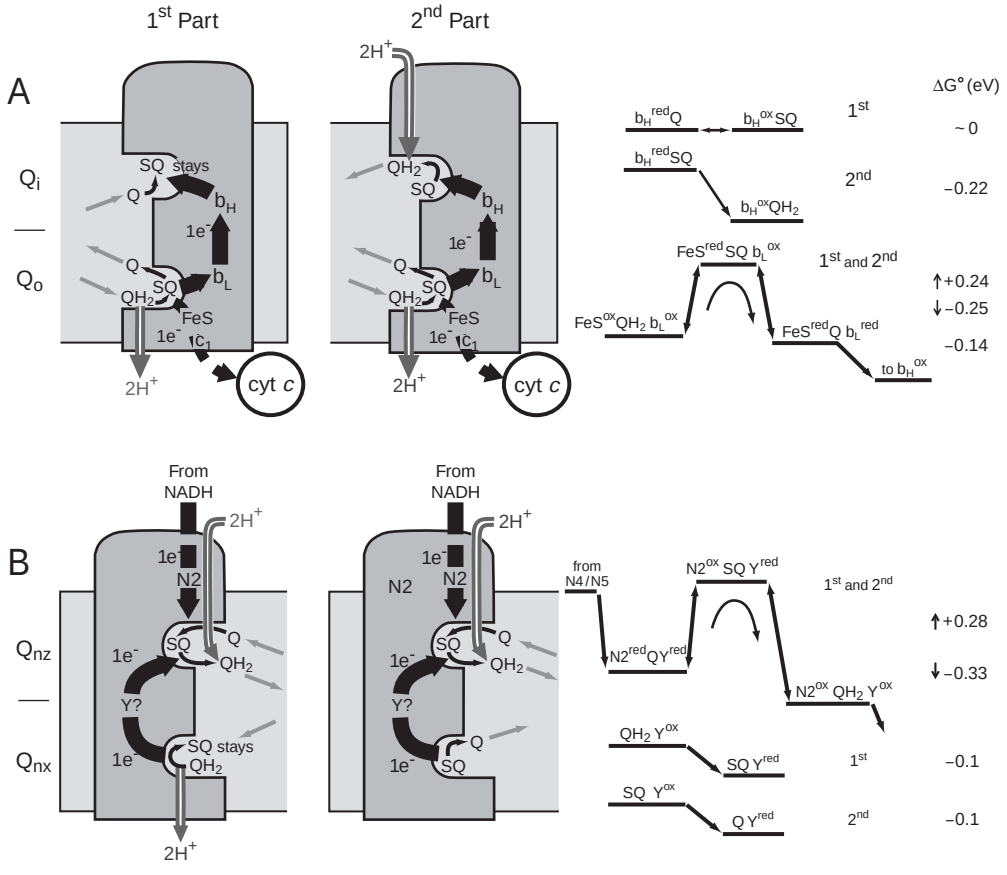

FIGURE 4.3 The two parts of energy conversion mechanism of Complex III and an analogous hypothetical model for Complex I. In A, in order to complete the turnover of the modified scheme, the Q_o site of the Complex III must oxidize two QH_2 molecules and the Q_i site reduce one Q molecule. Thus the overall reaction is one QH_2 oxidized *net* and two ferric cyt *c* hemes reduced. The figure tracks the stoichiometries of electrons, protons, and charges in each of the two parts. On the right are shown the energy profiles of the reaction's potential values listed in Table 4.1. Note, for simplicity, only the Q_{os} data discussed later is included. In B, the analogous reactions are shown for Compex I; in the energetics profile, a potential of −0.05V is chosen for N2.

the inset better represents the physical relationships of the five ubiquinone catalytic sites with the membrane pool. The various fluxes of Q and QH_2 in three arms share the same space of the membrane ubiquinone pool. While the Complex I model remains hypothetical, it is nevertheless a testament to scientific perseverance that for over 40 years, Mitchell's early problematic failure to account for two out of three 2H carrying arms has only recently acquired such a complete and elegant working solution.

Elegant as the solution may be, compared to the simple linear scheme of Figure 4.1, that outlined in Figure 4.2 requires a bit more thought and very major modifications in mechanistic principles. No longer is respiratory energy conversion a simple matter of smooth linear flow of "2e-" and 2H transfer zig-zagging the membrane. Complexes III and I now possess localized sites of energy conversion, namely the Q_o and Q_{nz} sites respectively, where the full extent of the free energy of the substrate oxidant and reductant (NADH-Q and QH_2-ferric cyt c) are brought to bear. Also, when considering mechanistic models of the kind shown in Figure 4.2, electron transfer must be considered in terms of singular events. Figure 4.3A shows the well-supported, and currently generally accepted single electron and proton pathways in Complex III. The process can be broken down into two parts. In the first part, a QH_2 from the pool binds to the Q_o site. One electron is drawn from

the QH_2 into a roughly isopotential high potential redox chain (Table 4.1) starting with an oxidized [2Fe2S] cluster and then cyt c_1 and cyt c at the interface. This chain delivers the full oxidizing potential of substrate ferric cyt c to the QH_2 in the Q_o site to provide enough initial energy to catalyze thermally activated endergonic electron transfer to reach the highly reactive SQ in the site;[20] see the energy schemes on the right that are drawn from the redox potential data in Table 4.1. The SQ so formed in this transition state[21,22] promptly reduces the nearby first member of a low potential chain, cyt b_L. Two protons are released from the Q_o site and the product Q is free to move into the pool. The low potential chain, driven by a free energy drop of −0.14eV between cyt b_L and then cyt b_H, promotes electron transfer across the membrane to the Q_i site where a Q from the pool is reduced to its Q_i site SQ state. As is indicated, free energy profiles on the right, the potentials of cyt b_H and the SQ/Q couples are about the same, so the electron is shared.[23] In contrast to the Q_o site, but similar to the Q_B site of the reaction center,[24] this reaction is feasible because the *S*Q is stabilized by the Q_i site and, as a result, stays bound for a sufficient time to allow the second part to proceed. In the second part, the process is repeated with only one major difference. In the second part, the SQ in the Q_i site is firmly reduced to QH_2 with the binding of 2 protons from the matrix.[23]

TABLE 4.1
Measured and Suggested Redox Properties of Mainly Beef Heart Mitochondria Respiratory Chain Components

Complex	1 Eectron Redox Center	E_{m7} (V)	2 Electron Redox Center	E_{m7} Q/SQ (V)	E_{m7} SQ/QH_2 (V)	E_{m7} ave(V)	log K_{stab}
I	[a]FeS N1a	−0.38	[a]FMN	−0.389	−0.293	−0.34	−1.6
I	FeS N1b	−0.25	Q_{nx}[b]	0.09	0.09	0.09	0
I	FeS N2	−0.15 to −0.05	Q_{ny}[b]		0.19	—	>14
I	FeS N3	−0.25	Q_{nz}[b]	−0.33	0.52	0.09	−14
I	FeS N4	−0.25					
I	Fes N5	−0.25					
			[c]FAD	−0.127	−0.031	−0.08	−2
II	[c]2Fe—2S	−0.005	Q_s[d,e]	0.14	0.08	0.11	1
II	4Fe—4S	−0.40		0.40	0.128	0.084	−2
II	3Fe—4S	0.06	Q_s[d,f]			—	
III	Cyt b_L	−0.09	Q_i[g]	0.030	0.27	0.15	−4
III	Cyt b_H	0.05	Q_{os}[g]	−0.33[i]	0.52[j]	0.09	−14
III	[2Fe-2S][h]	0.28	Q_{ow}[g]	−0.22[i]	0.38[j]	0.08	−10
III	Cyt c_1	0.23					
Diffuser			NAD	−0.92	0.28	−0.32	−20
Diffuser			Q_{pool}[i]	−0.23[j]	0.39[j]	0.09	−10[j]
Diffuser	Cyt c	0.28					
			Succ/Fum	[k]	[k]	0.025	[k]

[a] see [9] for review of iron sulfur components and E_{m7} values
[b] components and E_{m7} values hypothetical
[c] see [62–64]
[d] the Qs site includes two ubiquinones with identical properties
[e] in submitochondrial particles, pH 7.4 [65, 68]
[f] in isolated succinate ubiquinone reductase, pH 7.4 [68]
[g] data from *Rb. capsulatus* [23]
[h] see [9]
[i] data from *Rb. sphaeroides* [39] *Rb. capsulatus* [40]
[j] estimate
[k] unknown

In comparison to the Q_o site, the energetics of this process shown on the right appears relatively straightforward. Although it is well established that 2.0 ± 0.1 H+ are bound as described by Complex III (see, for example, 25), there is hardly any information at the molecular level on the proton movement in the protein itself, or release at the Q_o site (see 26, 27). This is in stark contrast with the work on reaction centers.[24]

Figure 4.3B shows the hypothetical model for Complex I in which electron and proton pathways can be traced in the same way as Complex III.[12] The principal difference is that the initiating single electron comes from the NADH redox chain ending with N2, which singly reduces a Q drawn from the pool into the Q_{nz} site to form an SQ. As with the Q_o site, the SQ is unstable but in contrast to the Q_o site, it acts as an oxidant to its adjacent redox center (shown as "Y"; Q_{ny} in Table 4.1, see later) to become QH_2. Electrogenic electron transfer across the membrane follows in a manner vectorially identical to that of Complex III. The Q_i site analogue, Q_{nx} site is predicted to also stabilize the SQ so that the QH_2 oxidation catalyzed by the Q_{nx} site can occur in two distinct steps as shown. Suggested midpoint potentials are listed in Table 4.1 and the energetics are shown on the right.

One more layer of modifications is needed before we reach contemporary views of respiratory electron and proton transfer and energy conversion; again, ubiquinone is a possible player in these novel proposals. The model needs to be augmented in light of the escalation from 6 to, at the least, 10 in the total number of protons and charges found to be translocated across the membrane per movement of two electrons from NADH to oxygen. Two of these extra protons and charges were found by Wikstrom to be associated with cytochrome oxidase some 20 years ago.[28] Evidence continues to accumulate to strongly suggest that charged proton ions (2 protons and $2q^+$ together) are translocated in an oxygen-driven proton pumping action that operates in addition to the traditional electrogenic arm for O_2 reduction to yield the observed total of 2 protons and $4q^+$ per 1/2 O_2 reduced in cytochrome oxidase. The other two protons and charges have been identified with Complex I,[29–35] which obviously challenges the model of Figures 4.2 and 4.3B. In order to double the number of protons and charges translocated per NADH oxidized and Q rescued with the rather limited components available in Complex I, we have borrowed ideas from Complex IV and proposed an additional role for Q in the electrogenic reaction of Complex I as is shown in Figure 4.4. We suggest that in the electrogenic step of Complex I, there is an analogous

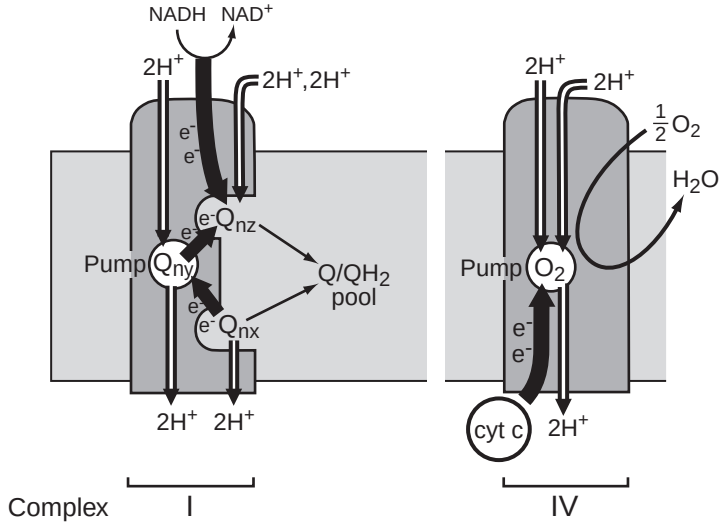

FIGURE 4.4 Proton pumps in Complex IV and an analogous hypothetical model for Complex I. The finding of an increased number of protons and charges in the respiratory chain determined that the two extra translocated protons/charges were associated with a localized molecular proton pump in Complex IV. We suggest that a similar pump in the Q_{ny} site might exist in Complex I to also explain the two extra translocated protons/charges. The position of the proposed Q_{ny} site is also necessary to facilitate electron transfer across the membrane.

additional proton pump element mediated by a permanently bound ubiquinone. The pump is situated between the Q_{nx} and Q_{nz} sites and is called the Q_{ny} site. This purely hypothetical construct raises the number of H^+ and charges translocated by Complex I to nicely match the findings that close to 4 protons and $4q^+$ are translocated in Complex I. It should be mentioned that despite a great deal of experimental work done and viable models proposed, the details of the molecular mechanism of the oxygen driven pump in Complex IV remain elusive;[36–38] a greater challenge awaits those working on the proton translocation mechanisms in Complex I.

4.3 UBIQUINONE CATALYSIS, SEMIQUINONES, SITE STRUCTURES, AND REGULATION IN ENERGY CONVERSION

It is well known that many substrate redox couples such as $NADH/NAD^+$, succinate/fumarate or ubiquinone QH_2/Q in solution are remarkably resistant to chemical oxidation-reduction by adventitious oxidants and reductants. Such stability is commonly attributed to the highly cooperative two-electron transition ($n = 2.0$) between their oxidized and reduced forms, typified by Eq. 4.2 for Q and QH_2, in which the highly reactive, singly reduced ubisemiquinone (SQ) state is always at vanishingly low concentrations. Oxidation or reduction of quinone in solution is slow, because an encounter with a single electron oxidant or reductant will only rarely generate the SQ state, and even more rarely will this SQ state encounter another oxidant or reductant to complete the net 2-electron transfer. Mitchell estimated the SQ to be maximally present in ubiquinone solutions at equilibrium at about 1 part in 10^{10} and so, not surprisingly, the SQ has not been detected to date. The absence of a detectable characteristic SQ radical g = 2.0045 EPR signal has pushed the limit of the SQ stability down to about 1 part in 10^7. The actual stability of the SQ of ubiquinone itself remains unmeasured, but it is likely to be very low, perhaps lower than Mitchell's estimate of the semiquinone stability constant (K_{stab}) of 10^{-10} given by Eq. 4.2:

$$QH_2 + Q = 2SQ; \qquad K_{stab} = [SQ]^2/[QH_2][Q] \tag{4.3}$$

The most reliable investigations aimed at determining the redox potential of the QH_2/Q couple in the Q_{pool} in membranes suspended in aqueous media has not been done with mitochondria. Detailed measurements have been done on photosynthetic bacterial membranes of *Rhodobacter sphaeroides* and *Rb. capsulatus*, yielding the same value of $+0.09 \pm 0.01$ V at pH 7.0 (E_{m7}); 2 protons and 2 electrons were demonstrated to have been exchanged from pH 5 to 9 in line with Eq. 4.2.[39,40] To our knowledge, ubiquinone has never been measured in mitochondrial membranes by a reliable potentiometric method. Earlier values determined for mitochondrial ubiquinone were somewhat lower (~ + 0.06 V) and almost certainly influenced by the method of measurement.[41] Nevertheless, the value obtained for ubiquinone with bacteria is most probably close to that in mitochondria. Although the redox potential values presented in Table 4.1 for ubiquinone in the membrane and in the various sites are not all from mitochondria, the detailed potential values of ubiquinone in analogous sites from different biological species will be close enough not to impact on general mechanistic considerations at the present stage of development. We can divide the ubiquinone sites so far discussed into three broad classes based on both the K_{stab} and the functional role.

4.3.1 Sites of Primary Energy Conversion: SQ Not Significantly Stabilized—K_{stab} Low

These sites are at the heart of energy conversion in the Q_o site in Complex III and the proposed Q_{nz} site in Complex I. These sites require a combination of the low K_{stab} of the SQ in the site and a remarkable cooperation of the two, one-electron redox centers, which must closely flank the site. It is this arrangement that gives rise to the well-known phenomena of "oxidant-induced reduction"

catalyzed by the Q_o site in Complex III: oxidation of cyt *c*, and hence [2Fe2S], causes the reduction of cyt b_L. We predict that the Q_{nz} site will operate similarly to catalyze the "reductant-induced oxidation," that is reduction of the N2 cluster will elicit the oxidation of QH_2 in the Q_{ny} site.[12]

The emerging structures of Complex III from several research groups[42–45] are an exciting development. Unfortunately, no ubiquinone has yet been identified in the key energy converting-Q_o site of any of the crystal structures to date. The ubiquinone complement in many cases has dissociated from the isolated complexes during isolation or, if present, may have become disordered[46] during the crystallizing procedures. However, the location of the Q_o site, like that which presaged the identification of the Q_i site occupant, has been deduced from the clear localization in the structures of the headgroups of the strongly binding inhibitors such as stigmatellin or the methoxyacrylates that readily cocrystallize with Complex III. Extensive biochemical studies have demonstrated that stigmatellin closely interacts with the [2Fe-2S] cluster and raises its potential over 0.2 V.[47] Methoxyacrylates are positioned close to cyt b_L (see refs [48–51] for discussions and earlier references). The x-ray structures provide vivid views of earlier models of the Q_o site operation discussed above, supporting the requirement that the Q_o site ubiquinone must be flanked by the [2Fe-2S] cluster and cyt b_L. However, the structures also gave us something completely unexpected. The [2Fe2S] cluster subunit appears in different locations in the various x-ray structures. It seems likely that the [2Fe2S] cluster subunit flanking the Q_o site actually rotates through about 55° and translates the cluster over a distance of about 10 Å between the ubiquinone of the Q_o site and the cytochrome c_1. This has been collectively considered by the crystallography groups to perhaps be an essential component of the regulation of the remarkable bifurcation of the two electrons from QH_2 in the Q_o site, one along the high potential chain in one direction and one down the low potential chain in the other.

Recent experiments done on mutants of *Rb. capsulatus* have revealed and time-resolved the motion[52] of the [2Fe2S] cluster subunit in the primary energy conserving steps of Complex III. If we apply electron tunneling calculations[20,53] to the Complex III structures [see 52] they clearly show that for electrons to pass from the QH_2 at the Q_o site to cyt c_1, the intervening [2Fe2S] cluster must move. The calculations and measurements[52] also show that the motion could be an essential mechanical regulator, which may be likened to the escapement mechanism in clocks. The mechanism is based on the large difference (for 10Å, about a 10^6-fold[20,53]) in electron transfer rates from the QH_2 to the [2Fe2S] cluster when proximal to the Q_o site or when proximal to the cyt c_1 heme. The controlled rate of transit in microseconds time is viewed to regulate the transfer of one electron out of the QH_2 giving time for the electron on the SQ to escape through cyts b_L, b_H, and the Q_i site across the membrane to usefully generate $\Delta\psi$. Figure 4.4 shows how the escapement might work; it is quite reversible.

We have searched for evidence for a similar action in the analogous [4Fe4S] cluster subunit, N2 of Complex I, and find encouraging hints that it may also move. For instance, like the [2Fe2S] cluster of Complex III, the N2-containing subunit, either TYKY or PSST, is located close to the membrane aqueous interface[11,54] and has approximately the same molecular size (20 kDa). The N2 similarly displays pH dependent redox potentials and has a long history of variable values (−0.050 to −0.150V).[55] Moreover, it displays sensitivity to the inhibitor rotenenone, which causes a 0.05V positive shift in the potential, [T. Ohnishi personal communication] perhaps in line with that of the [2Fe2S] cluster, the potential of which is very sensitive to additions of stigmatellin.[47] N2 displays multiple spin couplings with radicals considered to be adjacent SQ states,[56,57] which could belong to the Q_{ny} at various distances or, through it, to the Q_{nx} site. In this speculation, as with the [2Fe2S] cluster of Complex III, the N2 subunit would move between an adjacent low potential iron-sulfur cluster, perhaps N4 or N5,[58] and the ubiquinone bound to the Q_{nz} site. In the same way as indicated in Figure 4.5 a regulated, escapement-like reduction of the Q_{nz} site Q may occur with one electron received from the NADH iron-sulfur redox chain and one received from the Q_{ny} site essential for energy conversion in Complex I.[12]

The most thorough kinetic, thermodynamic, and biochemical and molecular biological work done on primary energy converting quinone sites has been with the Q_o site of the Complex III of

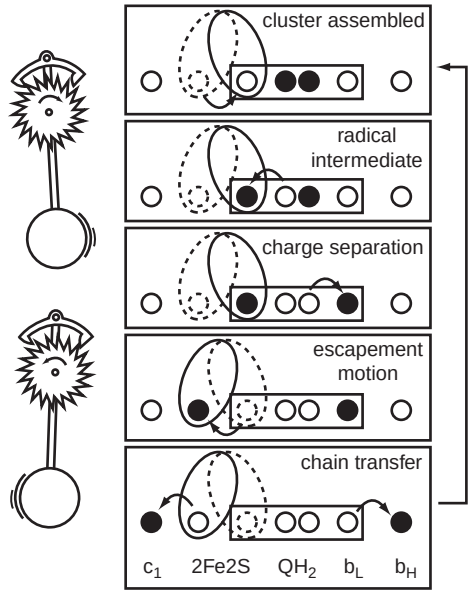

FIGURE 4.5 An escapement mechanism for regulating QH_2 and Q oxidation reduction in Q_n site energy conversion.

Rhodobacter capsulatus. These studies suggest that there are in fact two ubiquinone binding domains, called the Q_{os} and Q_{ow} domains for their strong and weak binding interactions with the Q_o site.[22,48–51,59] The occupant of the Q_{os} domain is characterized by a very slow exchange with the Q_{pool} while the Q_{ow} domain supports rapid exchange of Q and QH_2 with the pool. The midpoint potentials of the ubiquinones of both domains in the bacterial Q_o site are within 0 to 0.15V of that of the Q_{pool} showing little or no alteration of the average potential upon binding to the site, and hence little or no functionally significant preferential binding affinity of the site for either Q or QH_2. Not surprisingly, no SQ states have been assigned to the Q_o site. Only recently has the degree of mitochondrial Q_o site stability been clarified. Experiments done in the early 1980s[60] showed that under turnover conditions, a radical species assigned to this site could easily be detected. However, recent work[61] has demonstrated that this radical was not associated with the Q_o site, but with the Q_s site associated with Complex II. This result is consistent with many redox titrations done on the ubiquinone of the Q_o site. Furthermore, examination of Complex III of *Rhodobacter capsulatus* by EPR under equilibrium conditions suggests the K_{stab} values are lower than 10^{-7}.[39] More recent estimates based on functional considerations put them much lower than this, at 10^{-10} for the Q_{ow} site and 10^{-14} for the Q_{os} site ubiquinones;[22] these are the values used in Figure 4.3A and in Table 4.1. However, further considerations based on our electron tunneling rate calculations[20] suggest that the K_{stab} values could be as low as 10^{-24} before adversely impacting on the rate of Q/QH_2 redox catalysis (k_{cat} 1700 s^{-1}) by the Q_o site. Table 4.1 presents suggested properties of the hypothetical Q_{nz} site proposed to fulfill the same primary energy converting steps in Complex I.

4.3.2 Sites of Secondary Energy Conversion: SQ Stabilized Significantly—K_{STAB} between 10^{-4} and 10

The engineering tolerances for the operation of these secondary sites are much greater than evident in the sites typified by the Q_o site. The archetype of this class is that of the ubiquinone in the Q_B site of the bacterial photosynthetic reaction center, which has enjoyed 20 years of detailed physical, chemical, and more recently, high resolution structural investigation.[24] In this class the effect of

binding ubiquinone to a binding site is sufficient stabilization to catalyze the normally Q/QH_2 transition in two sequential $n = 1$ steps, as indicated for the Q_i site in Figure 4.3A. The midpoint potentials of the individual $n = 1$ steps (Q/SQ and SQ/QH_2) of this class are usually not far (< 60 mV) from that of the Q_{pool}, as listed for the Q_i and the Q_s site of Complex II in Table 4.1. The position of the quinone headgroup of the Q_i ubiquinone near the matrix side of the crystallized Complex III[42–45] was first roughly located by the easy visualization of the inhibitor antimycin, long considered to displace the Q of the Q_i site and well known to interact with heme b_H in the cytochrome *b* subunit. Indications of the weak but clear electron density of a ubiquinone headgroup close to the heme b_H is emerging. The analogous but still hypothetical Q_{nx} site is predicted in our proposal to exhibit the same properties and perform the same, although functionally reversed in Complex I; these are listed in Table 4.1.

The much simpler Complex II[62–64] possesses a long single electron redox chain starting with FAD at the catalytic site of fumarate/succinate oxidation-reduction followed by three iron sulfur clusters leading to the Q_s site in contact with the Q_{pool} in the membrane. The site has been shown to contain a pair of ubiquinones that possess quite stable SQ states with K_{stab} values in the 10^{-2} to 10^{1} range.[65–68] The strong spin–spin interaction of the SQ states in the two ubiquinones has enabled an estimate of their proximity at about 8Å edge-to-edge.[69] Similarly, both spins interact with and are relatively close to the nearest of the three iron-sulfur clusters. Whether one or both Q_s site ubiquinones exchange with the Q_{pool} or whether there is another site akin to the Q_{ow} domain of the Q_o site that rapidly exchanges Q/QH_2 with the pool, is not presently known. While no structure for Complex II is in hand, there is a structurally and functionally related one of the *E. coli* fumarate reductase that possesses two structurally different menaquinones in the membrane spanning region.[70] Moreover, the structure demonstrates that the possibility of three, in-sequence ubiquinone catalytic sites, as proposed to span the membrane profile in Complex I, is not so far-fetched.

4.3.3 Sites With SQ Greatly Stabilized—K_{STAB} Perhaps $> 10^4$

In the most extreme cases, the bound quinone uses only one redox couple to guide single tunneling electrons through protein to specific destinations as seen in the Q_A sites of bacterial and plant (PSII) photosynthetic reaction center.[19] The hypothetical Q_{ny} site suggested for the pump element in Complex I would be a member of this class. Q_{ny} would be permanently bound, like Q_A. However, while the Q_A site operates strictly between its ubiquinone and ubisemiquinone anion states, with the semiquinone anion highly stabilized and the QH_2 suppressed by the site structure, it is proposed that the Q_{ny} site will operate strictly between its hydroquinone and semiquinone states, with the presence of the quinone state suppressed. In Figure 4.4 we have suggested that the transition involves the exchange of one proton, although the possibility of two protons has been considered.[9] See Table 4.1 for the suggested redox state and midpoint potential and reference [9] for further details.

4.4 ENERGETICS

Table 4.1 and Figures 4.3 and 4.4 provide the basic information that makes 10 protons and 10 charges fit well into an efficient respiratory chain and, for our point of focus here, why for Complex I, II, and III, the 8 protons and 6 charges translocated fit so well into the highly reversible and major segment of the chain between the substrates. Figure 4.6 summarizes our discussions of the developing mechanistic roles of ubiquinone and focuses on the central position of ubiquinone in the overall thermodynamics of these three complexes. Thus, as obtained from Table 4.1, the midpoint potential difference (ΔE_{m7}) between the $NADH/NAD^+$ (−0.32 V) and QH_2/Q (+0.09 V), between succinate/fumarate (+0.025 V) and QH_2/Q, and between QH_2/Q and ferro/ferri cytochrome *c* are quite different: 0.41 V, 0.065 V, and 0.18 V, respectively. As indicated in the legend of the figure, because two electron equivalents are transferred to and from QH_2/Q, the $\Delta G°$ values for the redox spans of each site are −0.82 eV, −0.13 eV, and −0.36 eV per mole of Q reduced or QH_2 oxidized. The other critical component of the energetic description of the respiratory action is the transmembrane

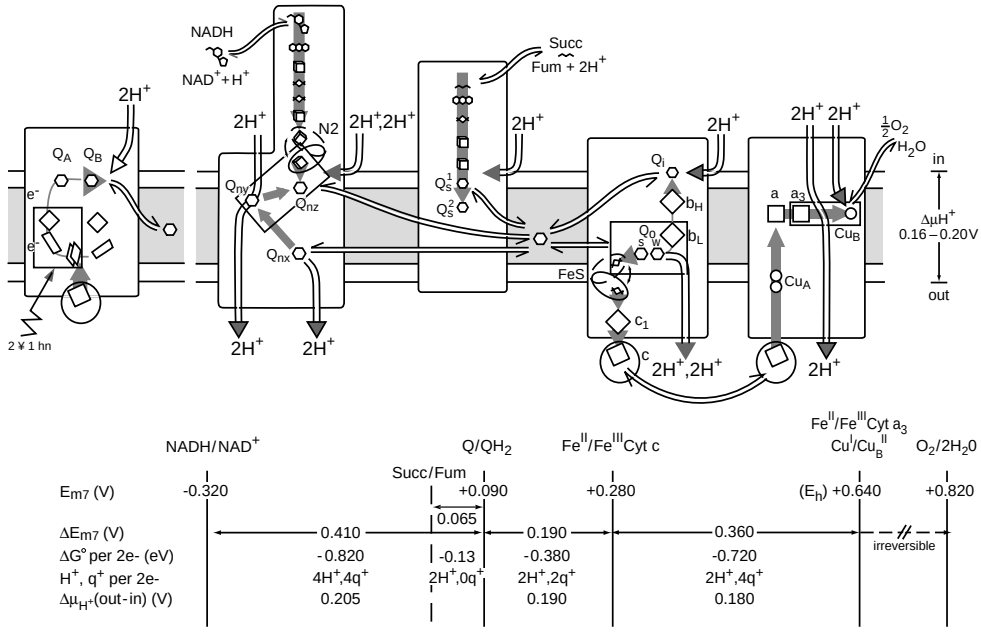

FIGURE 4.6 Contemporary working model of the roles of ubiquinone in electron and proton transfer and energy conversion in the mitochondrial respiratory chain. The figure incorporates the component parts discussed in the earlier figures and throughout the text. The energetics are taken from the substrate potentials of Table 4.1. The areas identified on Complexes I, III, and IV circumscribe the regions of the complexes identified with primary events of energy conversion; these events are localized and in principle not much different from the primary events of light energy conversion in the photosynthetic reaction center shown on the left. For the potential and energy scales at the bottom $-\Delta G^{\circ} = n_e \Delta E_{m7}$ in eV where n_{e-} represents the number of electrons transferred per mole of NADH or succinate oxidized or $\frac{1}{2}$ O_2 reduced, in this case 2.0; the scale is multiplied by 23.06 kcal per volt equivalent to convert into kcal/mole.

Note: Symbols used to represent the redox cofactors are as follows: hexagons, ubiquinone; three small fused hexagons, flavins; cubes, [4Fe4S] or [3Fe4S] clusters (see Table 4.1); small diamonds, [2Fe2S] clusters; circles, copper atoms; and squares in various orientations, hemes in the respiratory complexes or chlorins in the reaction center.

electrochemical potential ($\Delta\mu_{H+}$) across the inner mitochondrial membrane. A large number of measurements done of the optimal $\Delta\mu_{H+}$ values achievable fall in the range of 0.16 to 0.19V. This is usually taken to comprise a ΔpH (out-in) of about 0.5 units (equiv. 0.030V) and the $\Delta\psi$ (out-in) of 0.12 to 0.16V. While the component values of the ΔpH and $\Delta\psi$ can vary widely depending on metabolic conditions and physiology and biological species, the total $\Delta\mu_{H+}$ values remain faithful to the widely different ΔG° value spans of each complex in the respiratory chain and the number of protons and charges translocated (N_{H+}) according to Eq. 4.4.

$$\Delta\mu_{H+}\ (\text{in-out}) = -\Delta G^{\circ}/N_{H+}\ (\text{V}) \tag{4.4}$$

If we look first at Complex III, perhaps the most secure in its experimental characterization, operating with a ΔG° of -0.36 eV (i.e., assuming for simplicity that the QH_2/Q_2 and ferro/ferri cyt c couples are operating at their midpoint potentials) we find that for 2 protons and 2 charges translocated per QH_2 oxidized by two cytochromes c, a $\Delta\mu_{H+}$ of 0.18V can be generated, nicely within the range of measured $\Delta\mu_{H+}$ values. The variance about this value can easily be accounted for by variances that only differ slightly from the biological "standard" states of UQ and cyt c defined by the ΔG° and ΔE_{m7} values. For instance, for a prevailing $\Delta\mu_{H+}$ value at the lower end

of the range (0.16 V), the ΔE_m between the QH_2/Q couple and ferro/ferricyt *c* would be similar and hence (20 mV) smaller than indicated by the standard states. Complex II, operating between succinate/fumarate and QH_2/Q (ΔE_{m7} 0.065 V and $\Delta G°$ −0.13 eV) can indeed easily translocate two hydrogens to help develop a ΔpH equivalent to −0.06 eV (or a ΔpH of one unit) and operate comfortably to meet the energetic requirements without moving far away from their respective E_m values. The same thinking must apply to Complex I with its $\Delta G°$ that is over ten times the value available to Complex II and more than double that of Complex III. The $\Delta G°$of −0.82 eV can comfortably accommodate the translocation of 4 protons and 4 charges per NADH oxidized and Q reduced. Indeed at a prevailing $\Delta\mu_{H+}$ value of 0.18V, as discussed for Complex III, 4 protons and charges translocated would require 4 times 0.18V or −0.72 eV before matching the prevailing $\Delta\mu_{H+}$, again leaving a $\Delta G°$ of 0.1 eV to spare.

It might be asked why Complex I does not individually operate at the higher values of the $\Delta\mu_{H+}$ range, to its maximum $\Delta\mu_{H+}$ value of 0.205V (i.e., 0.82 eV divided by 4) or whether Complex I can perhaps accommodate the translocation of a fifth proton and charge, or whether Complex II could drive the translocation of hydrogens to a ΔpH of one unit instead of the commonly measured 0.5 units or drive twice as many hydrogens. It is, however, possible that the lower $\Delta\mu_{H+}$ prevailing value may be dictated by the smaller energetic span of Complex III or by the individual kinetics of each Complex. But it should be remembered that this discussion is only applied to the "standard" state and when under operating conditions, it is the E_h values and ΔG that matter. It is equally important to recognize that the potentials and free energy differences and proton/charge stoichiometry variances that we are discussing are rather small and at the uncertainty limits of measurement in a very complicated system. The development of methods to realize more precise measurements is certainly justified, but for now the picture presented in Figure 4.5 is a very good approximation.

4.5 PROPERTIES AND POTENTIAL REACTIVITY OF UBISEMIQUINONE RADICALS WITH MOLECULAR OXYGEN

Table 4.1 summarizes the redox potentials of ubiquinone in the pool and the various catalytic sites in Complexes I–III and compares them with the aqueous properties of the lower redox states of oxygen. The ubiquinones in sites such as the Q_i, Q_{nx}, and Q_s sites that are involved in the simple interfacing of single electron transfer chains with the Q_{pool} display mildly reducing and oxidizing redox potentials not far from the average value of the Q_{pool}. More striking are the low and high redox potential values associated with the single electron couples involving Q/SQ and SQ/QH_2 in the Q_o site of Complex III and the hypothetical Q_{nz} site of Complex I sites where the ubiquinone is central to the primary steps of energy conversion. These SQ states do have the thermodynamic potential to reduce molecular oxygen to the superoxide state. And, although the ubiquinone SQ states must be short lived in the energy conversion process, there is the finite possibility that following O_2 reduction to the superoxide, further interactions ensue that lead to a cascade of damaging oxygen and other ubiquinone radical states emanating from these catalytic sites. The table provides us with the sources and the basis for understanding superoxide generation from Complex I and III of the kind first described by Chance and Boveris, 25 years ago.[71,72]

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5 Extramitochondrial Functions of Coenzyme Q

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CONTENTS

5.1 INTRODUCTION

Biological molecules can be considered as "nanomachines" that carry out all the physiological functions needed in the living cell. These molecules are organic compounds that can form complex macromolecules in most cellular components. Although organic compounds are relatively stable in anaerobic environments, oxygen can combine with most of them causing an oxidative alteration. In the vast majority of cases, this oxidation produces the partial or total inactivation of the biological molecules.

Cells have developed different defensive systems to survive in the presence of oxygen and its highly reactive species (ROS), in other words, systems that prevent the oxidation of their organic molecules. These mechanisms include antioxidant enzymes and small water- and lipid-soluble molecules able to be oxidized, rendering stable redox species.[1] Among these, coenzyme Q (CoQ, ubiquinone) is the only lipid-soluble antioxidant that is present and can be synthesized in all organisms studied thus far.[2] The ubiquitous presence of this molecule in cellular membranes suggests a very important role in the cell physiology.

Writing about CoQ has, for many years, been synonymous with writing about the electron transfer and energy transduction in the inner mitochondrial membrane. However, that point of view has been widened with the evidence that CoQ is also present in serum lipoproteins, endomembranes, and the plasma membrane in animal cells.[3] Plasma membranes from yeast cells also have a substantial amount of CoQ.[4] However, the plant plasma membrane apparently lacks CoQ. Instead, the naphtoquinone vitamin K might substitute for the extramitochondrial role of CoQ in higher plants.[5]

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Ernster and Beyer[6] have extensively documented that CoQ acts as an antioxidant within extramitochondrial membranes. This role for CoQ was completely accepted when it was demonstrated that CoQ is not physiologically isolated, but related to other redox molecules. Different authors reported that CoQ keeps other antioxidants reduced such as vitamin E[7,8] and vitamin C.[9] Moreover, CoQ can be reduced by several extramitochondrial NAD(P)H-dependent enzymes.[10–13] This electron transport upstream and downstream of CoQ draws a meaningful picture for CoQ as a nonmitochondrial antioxidant integrated in the cell physiology.

CoQ is unevenly distributed among the different cellular membranes.[3,14] This could be a consequence of its proposed diversified roles in different membranes.[15,16] Recent results indicate that oxidative stress causes changes in the intracellular distribution of CoQ in mammalian and yeast cells (see [17, 18] and balance of this chapter). This redistribution suggests that CoQ content may not be a feature dependent on the membrane location. On the contrary, the membrane content of CoQ could be the consequence of the physiological status of the living cell.

The involvement of extramitochondrial CoQ in the defense against oxidative stress explains the enhanced resistance to undergoing apoptosis in serum-depleted, CoQ-enriched cells.[17] This enhancement is similar to that produced by other antioxidants such as ascorbate or vitamin E using similar whole cell systems.[19,20] Furthermore, an *in vivo* model using Long-Evans rats definitely demonstrates the cooperation between vitamin E and CoQ in the defense against oxidative stress.[21] The control of cell growth and differentiation also has been related to the cellular redox status. CoQ, as well as ascorbate, also has influence on cell growth.[22,23] The supplementation of cell culture media with CoQ leads to an increase of cell growth and also partially prevents oxidative stress-induced apoptosis.[20,24]

In this chapter, we will present a detailed review of how extramitochondrial CoQ participates in nonmitochondrial membrane-associated redox activities and its consequences in several physiological processes such as the defense against oxidative stress or the control of growth and death of cells.

5.2 PRESENCE OF COENZYME Q IN EXTRAMITOCHONDRIAL MEMBRANES

The first complete study of CoQ distribution in endomembranes was carried out in subcellular fractions of rat liver hepatocytes. The results showed that CoQ appears unevenly distributed among cell membranes. Surprisingly, Golgi membranes and lysosomes contained as much CoQ as was found in inner mitochondrial membranes, followed by the plasma membrane, whereas much less CoQ was associated with the endoplasmic reticulum.[3] Interestingly, the concentration of CoQ in microsomes responds to stress in a different way than that of mitochondria.[15]

The analysis of the redox state of CoQ is helpful in indicating its possible functions in extramitochondrial membranes. The study by Takahashi et al.[14] has confirmed the evidence that all subcellular fractions contain significant amounts of CoQ, although the levels at the plasma membrane were higher than in previous studies. A significant portion of total CoQ (70 to 80%) was present in the reduced form (ubiquinol, $CoQH_2$) in most membranes, although this ratio was decreased to 30% in the plasma membrane.

CoQ has also been detected in the plasma membrane, mitochondria, and azurophilic granules of neutrophils.[25] While a function for CoQ in the generation of ROS was initially suggested,[26,27] it is now clear that NADPH-oxidase activity of neutrophils does not require CoQ.[28] More likely, CoQ might protect the plasma membrane from the reactive species generated by these cells. In the yeast *Saccharomyces cerevisiae*, CoQ has also been measured in the plasma membrane and levels change according to the yeast strain used, culture media, and the growth state.[4]

The uneven CoQ distribution not only suggests an important role in each membrane, but also indicates that there may exist a specific mechanism of synthesis and/or distribution. A key enzyme in CoQ biosynthesis—nonaprenyl-4-hydroxy-benzoate transferase—was found in several subcellular

fractions,[29] indicating a possible biosynthesis pathway common to the various compartments. This possibility was restricted to endoplasmic reticulum and Golgi membranes by later studies showing an interlocked synthesis between both fractions mediated by a midway fraction called SII or smooth II microsomes. This data, together with the high CoQ levels in Golgi membranes, suggested the sequence endoplasmic reticulum-Golgi as the way of synthesis and distribution of CoQ among cell membranes.[30] More recent studies of CoQ biosynthesis in *Saccharomyces cerevisiae* have challenged this interpretation and demonstrated that, at least in yeasts, the CoQ biosynthesis is restricted to the inner mitochondrial membrane, and likely catalyzed by an enzymatic complex.[31] This observation showed that CoQ biosynthesis is a more complex pathway that could have dissimilar mechanisms in different organelles.

Mechanisms for CoQ distribution still remain obscure, but recent evidence obtained by our group may serve as a starting point to analyze the relationship between CoQ distribution among membranes and the physiological state of the cell. Thus, the treatment of yeast cells with H_2O_2 produces an increase of CoQ at the plasma membrane and a decrease in the rest of the membranes. This effect may be due to a CoQ mobilization between membranes or to *de novo* synthesis. The existence of a unique CoQ synthesis location in yeasts and the inhibitory effect of H_2O_2 treatment over biosynthetic genes,[32] allow us consider CoQ mobilization as the most likely explanation for these results.

Another indication that an active mechanism exists to deliver CoQ comes from the use of yeast mutant strains defective in the CoQ biosynthesis pathway. A yeast strain harboring a point mutation in the COQ7 gene did not grow in culture media with nonfermentable carbon sources, even if supplemented with exogenous CoQ_6. However, in another mutant strain obtained by total deletion of the COQ7 gene (coq7Δ), the growth was restored by added CoQ_6. Interestingly, CoQ was incorporated into the plasma membrane in both strains, but was only delivered to the mitochondria in the coq7Δ mutant.[33]

5.3 BIOCHEMISTRY OF EXTRAMITOCHONDRIAL UBIQUINONE

As a result of its antioxidant function, CoQ becomes oxidized. However, a substantial amount is maintained in its reduced state in the plasma membrane and endomembranes,[14] and in plasma lipoproteins as well.[7] Furthermore, natural ubiquinones become reduced after dietary uptake.[34] Although it is generally accepted that oxidized CoQ is the final product of its biosynthetic pathway, the *de novo* synthesis of the hydroquinone has also been proposed to contribute, at least partially, to the high levels of $CoQH_2$ observed *in vivo*.[34,35] However, *de novo* synthesis cannot explain how $CoQH_2$ is maintained during oxidative challenge. Thus, it is clear that some enzymatic systems must operate for $CoQH_2$ regeneration from its oxidized or semioxidized quinone. In the mitochondria, this function is linked to the electron transport chain dehydrogenases and other enzymes as well.[2,35,36] Current evidence supports that, similar to the inner mitochondrial membrane, $CoQH_2$ regeneration in extramitochondrial membranes is accomplished by the electron transport linked to oxidation of pyridine nucleotides by flavodehydrogenases.

Several enzymes have been reported to function as CoQ reductases outside mitochondria; most of them were previously characterized as flavoenzymes.[2,37] They fall into two categories according to the reduction mechanism of the quinone: *one-electron CoQ-reductases*, which reduce CoQ in two *successive* electron transfers via a semiquinone intermediate (as is the case for mitochondrial NADH-CoQ reductase), and *two-electron CoQ-reductases*, which reduce CoQ directly via *simultaneous* transfer of two electrons to the quinone.

5.3.1 One-Electron Ubiquinone Reductases

Two extramitochondrial enzymes able to reduce ubiquinone through a one-electron mechanism have been described. Both are integral membrane proteins whose participation in microsomal

electron chains has been long recognized, namely the NADH-cytochrome b_5 reductase and the NADPH-cytochrome P450 reductase.

The NADH-cytochrome b_5 reductase has been found in the cytosolic side of all endomembranes and the plasma membrane, and in the erythrocyte cytosol.[9,11,38,39] The reductase was known primarily by its function in the reduction of microsomal cytochrome b_5 to transfer electrons to the nonheme iron fatty acid desaturase,[40] and as a methemoglobin reductase.[39] This enzyme has also been related to the detoxification based on cytochrome P450 as an alternative source of electrons.[10] In addition, cytochrome b_5 reductase reduces a variety of quinones by a one-electron mechanism to generate the corresponding semiquinones and hydroquinones in the absence of cytochrome b_5,[10] and has been proposed to be involved in $CoQH_2$ regeneration in the plasma membrane.[9,11,41] Incubation of pig liver plasma membranes with NADH results in the reduction of endogenous CoQ_{10} to $CoQ_{10}H_2$, an activity attributed to the NADH-cytochrome b_5 reductase.[42]

As a CoQ reductase, the enzyme displays maximal activity with CoQ_0, a hydrophilic CoQ analogue. Reduction of natural hydrophobic homologues with long isoprenoid side-chain such as CoQ_{10} requires reconstitution into phospholipid liposomes.[11] The quinone moiety of CoQ_{10} is freely movable in the lipid bilayers and thus, it can orient itself toward both sides of membranes,[43] (see [43] and the balance of this book). At the cytosolic layer, CoQ_{10} might be accessible to the catalytic domain of NADH-cytochrome b_5 reductase, similar to the reaction between $CoQH_2$ and cytosolic myoglobin.[44]

NADPH-cytochrome P450 reductase can also reduce quinones through a one-electron mechanism.[10] Evidence for the putative role of this enzyme as a CoQ-reductase arises from the ability of NADPH-driven electron transport to inhibit lipid peroxidation, possibly through antioxidant recycling in a process requiring CoQ.[36]

5.3.2 Two-Electron Ubiquinone Reductases

Two enzymes have been reported to reduce ubiquinone through a two-electron mechanism, both residing in the cytosol: DT-diaphorase,[12,45] and a distinct NADPH-dependent ubiquinone reductase.[13,46,47]

Cytosolic DT-diaphorase is an inducible enzyme that can reduce a great variety of quinones and other substrates as well, as it is very sensitive to low concentrations of dicumarol. Much is known about its biochemistry, mechanism, and molecular biology,[37,48,49] although its physiological role has still not been defined. Since this enzyme is particularly active in the reduction of hydrophilic quinones, but displays little reactivity towards hydrophobic ones, its putative role in reduction of extramitochondrial CoQ_{10} was not initially considered.[37] However, it has been recently reported that DT-diaphorase can maintain the reduced state of hydrophobic ubiquinones in phospholipid liposomes, thus promoting antioxidant function. Consequently, the suggestion was made that this could represent its actual role *in vivo*.[12,45] Although DT-diaphorase is a soluble protein, its assay requires detergent to achieve maximal activity, which might indicate that the enzyme can interact with membrane components located at the membrane-cytosol interphase.[12] In addition, a minor portion is usually associated with membranes,[37] which may be relevant for protection of extramitochondrial membranes against oxidative stress.[21]

In addtion to DT-diaphorase, a novel cytosolic NADPH-ubiquinone reductase has been recently described.[13,46,47] This enzyme can be distinguished from microsomal and mitochondrial enzymes, as well as DT-diaphorase, and its activity remains insensitive to low concentrations of dicumarol.[13,46] *In vitro* studies have demonstrated that this enzyme reduces CoQ in liposomes and microsomes and also inhibits lipid peroxidation in these membranes, acting preferentially on long-chain CoQ analogues, like CoQ_{10}.[13,47] Further studies have shown that this enzyme also functions in antioxidant regeneration in animals treated with carbon tetrachloride.[50]

5.3.3 Extramitochondrial Ubiquinone Oxidases

Ubiquinone participates as an electron carrier in transplasma membrane electron transport mediating the reduction of extracellular oxidants such as ferricyanide, diferric transferrin, and the ascorbate

free radical.[9,51] The exact mechanism for electron transfer from plasma membrane ubiquinol to extracellular oxidants has not been fully defined. Current evidence suggests that, unlike the direct chemical reaction observed between ubiquinol and the tocopheroxyl radical inside the membrane,[8,36] the reduction of extracellular oxidants requires additional enzymatic components facing the external side of the plasma membrane.[52–55] NADH-oxidase activity partially purified from the surface of HeLa cells has been recently reported by Kishi et al.[56] to exhibit $CoQH_2$-oxidase activity. Whether this protein is a terminal oxidase of CoQ-dependent transplasma membrane electron transport remains to be elucidated.

5.4 COENZYME Q AND THE PLASMA MEMBRANE REDOX SYSTEM

Several studies have shown the presence of significant amounts of CoQ at the plasma membrane of eukaryotic cells such as hepatocytes[3,14] and *Saccharomyces cerevisiae*,[4] which indicates an important role in the biochemistry and physiology of the plasma membrane. Several possibilities have been proposed to explain this presence; a storage for transfer to other compartments or blood serum,[3] a role as an antioxidant within the lipid bilayer,[8] and finally, acting at the plasma membrane as an intermediate electron carrier across the membrane as it does in mitochondria.[51]

This last aspect links the CoQ with the plasma membrane redox system. The plasma membrane contains several redox activities, some of them related to a transmembrane electron transport system that reduces external impermeable oxidants using intracellular electron donors such as NADH.[57,58] Although the physiological functions of the transmembrane redox system are still a matter of study, they have been related to the control of animal cell growth and differentiation.[57,59,60] Recent evidence supports the involvement of CoQ as an intermediate electron carrier in transplasma membrane redox activity.

The participation of CoQ as a component of the plasma membrane electron transport has been determined by several approaches including solvent extraction of the membranes to remove CoQ; the inhibition of electron transport by quinone antagonists such as capsaicin, chloroquine, and resinifera toxin; and nonfunctional CoQ analogues, which compete for the quinone site such as 2,3-dimethoxy-5-chloro-6-naphtylmercapto-1,4-benzoquinone and 2-methoxy-3-ethoxy-5-methyl-6-hexadecylmercapto-1,4-benzoquinone.[16,51] Plasma membrane redox transport is inhibited by removal of CoQ with heptane and the activity is recovered after CoQ_{10} restoration.[9,51,61] Inhibitory CoQ analogues also decrease electron transport in both isolated plasma membrane and whole cells, and CoQ_{10} reverses this inhibition. In addition, redox activity is increased by short chain CoQ analogues.[6,62]

The involvement of CoQ in the transplasma membrane redox system is supported by the different effect of CoQ on *cis*-oriented redox activities (donor and acceptor sites located at the same side of the membrane) as NADH-cytochrome *c* oxidoreductase, and on *trans*-oriented activities (donor and acceptor at opposite sides of the membrane) as NADH-ascorbate free radical (AFR) reductase. These experiments have shown that the *trans*-oriented activity can be modulated by CoQ, whereas *cis*-oriented activity remains insensitive to the CoQ status of the plasma membrane.[9]

Genetic evidence for the participation of CoQ in the plasma membrane redox system comes from the analysis of several redox activities in plasma membranes isolated with wild-type *Saccharomyces cerevisiae* and a *Coq3* mutant strain deficient in CoQ biosynthesis pathways. These studies have indicated that the plasma membrane of *Coq3* mutants, which absolutely lack CoQ, has extremely low levels of the *trans*-oriented activity NADH-AFR reductase, while NADH-ferricyanide and -cytochrome *c* oxidoreductase activities show only partial inhibition. Deficiency was abolished when mutant cells were cultured with exogenous CoQ, or when transformed with a plasmid harboring the wild type gene *Coq3*. AFR reduction did not involve superoxide since it was insensitive to SOD. However, superoxide produced by reaction of semiquinones with molecular oxygen accounted for most CoQ-dependent ferricyanide and cytochrome *c* oxidoreductases.[54] These results are apparently in contrast with those obtained with pig liver plasma membranes, where the

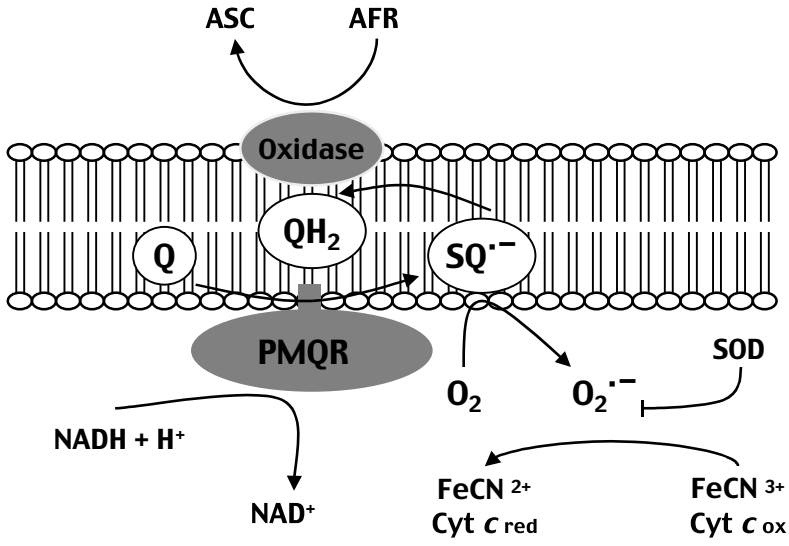

FIGURE 5.1 Role of CoQ in plasma membrane redox activities. The plasma membrane CoQ-reductase (PMQR) catalyzes the one-electron reduction of CoQ (Q) to ubisemiquinone (SQ^-) and then ubiquinol (QH_2). CoQ-dependent ferricyanide (FeCN) and cytochrome *c* (Cyt c) reductases are based on superoxide generation by reaction of ubisemiquinones with oxygen. Thus, both activities are sensitive to superoxide dismutase (SOD). However, ascorbate free radical (AFR) reductase is unique and not dependent on superoxide. Transmembrane NADH-AFR reductase possibly involves a yet unidentified oxidase facing the external side of the plasma membrane.

cytochrome *c* oxidoreductase is independent of the CoQ status,[9] (see above). This discrepancy may be explained by differences in the isoprenoid side-chain length of the corresponding CoQ homologues. It has been reported that rates of superoxide generation correlate directly with the content of CoQ_9 and inversely with that of CoQ_{10} in heart mitochondria of different mammals,[63] and the suggestion has been made that CoQ_9 and CoQ_{10} may play different roles as antioxidants or components of the mitochondrial respiratory chain.[64] This difference may be even more pronounced between plasma membranes isolated from pig (containing CoQ_{10}) and yeast (containing CoQ_6). A scheme showing the participation of CoQ in various plasma membrane redox activities is despicted in Figure 5.1.

The CoQ participation in the NADH-AFR reductase also indicates a role in the ascorbate stabilization. This activity was first shown in HL-60 cells,[65] and later recognized in several cell types as neuroblastoma,[66] retinoblastoma,[67] K-562,[61,68] and yeast cells. [4,69]

The relationship between CoQ and ascorbate regeneration was first evidenced in *Saccharomyces cerevisiae*, where an increase in ascorbate regeneration activity in cells supplemented with exogenous CoQ_6 was demonstrated,[69] and later confirmed with the use of yeast mutants deficient in CoQ biosynthesis.[4] CoQ supplementation also enhances ascorbate stabilization in K-562 cells and NADH-AFR reductase activity at the plasma membrane. Increasing the amount of the 34 kDa NADH-cytochrome b_5 reductase by liposome fusion stimulates ascorbate stabilization in whole cells.[61] This phenomenon finds a physiological parallelism in the increase of both CoQ content at the plasma membrane and ascorbate regeneration in K-562 cells observed during the TPA-induced differentiation process,[61] similar to that observed in yeast during the exponential-to-stationary cell growth transition.[4]

Several functions have been proposed for CoQ-dependent transplasma membrane electron transport, including the regulation of the cytosolic NAD^+/NADH ratio. The treatment of cells with ethidium bromide produces the loss of mitochondrial DNA and the mitochondrial function.[70] In Namalwa cells, this treatment leads to an activation of the plasma membrane redox system to reoxidize cytosolic NADH, which accumulates in excess, exporting reducing equivalents to external acceptors and thus equilibrating the NAD^+/NADH ratio toward normal levels.[71] Mitochondria

depletion of HL-60 cells with ethidium bromide results in an increase of CoQ at the plasma membrane and transmembrane redox activity measured as ascorbate stabilization.[23]

Another possible function of this CoQ-dependent transplasma membrane redox activity is the maintenance of an antioxidant system to scavenge the extracellular oxidants using cytosolic reducing equivalents exported by CoQ at the plasma membrane. The protective role of CoQ via ascorbate stabilization is supported by recent experiments carried out by our group with yeast cells exposed to high concentrations of H_2O_2 (2.5 mM). This treatment produces a severe growth arrest, CoQ accumulation at the plasma membrane, and a great increase in ascorbate stabilization.[72] This function, linked to the role of CoQ in the reduction of the tocopheroxyl radical to regenerate tocopherol at the lipid bilayer,[7,73] depicts a model for antioxidant protection where CoQ protects both the lipid- and the water-soluble phases of cells against oxidant compounds.

5.5 EXTRAMITOCHONDRIAL COENZYME Q IN THE DEFENSE AGAINST OXIDATIVE STRESS

Oxidative stress can be defined as the result of oxidative changes in the cellular redox status. This status is the consequence of the equilibrium between oxidants and physiological antioxidants. The main sources of oxidative stress are oxygen and its ROS. Extracellular ROS mainly come from the interaction between transition metal ions and oxygen in solution, or by the radiation-induced hydrolysis. The most important intracellular sources of ROS are oxygen-metabolizing organelles such as mitochondria and peroxisomes (see Kehrer and Smith for a in-depth review, Chap. 2 Nat. Antiox in health and disease).

In addition to its role as an electron carrier in electron chains associated with membranes, $CoQH_2$ is an important antioxidant that protects membranes from peroxidations.[6,35] As covered in detail in a next section of this book, antioxidant properties of $CoQH_2$ rely on a direct scavenging of initiators and lipid peroxyl free radicals, and its ability to regenerate other antioxidants. Extramitochondrial membranes have enzymatic systems that catalyze the reduction of CoQ to the semiquinone or hydroquinone using NAD(P)H (see above). Accordingly, NADH protects isolated plasma membranes, and proteliposomes containing CoQ_{10} and purified cytochrome b_5 reductase, against peroxidation initiated by thermal decomposition of 2,2′-azobis(2-amidinopropane) (AAPH).[74] These results demonstrate an antioxidant role for the cytochrome b_5 reductase via $CoQH_2$ regeneration at the plasma membrane. A similar role for the NADPH-cytochrome P450 reductase, possibly through antioxidant recycling in a process requiring CoQ, has also been proposed recently.[36,75] This protective action of NAD(P)H is in contrast with the prooxidant effect of NAD(P)H-driven electron transport in the presence of iron,[76] which can unmask its putative antioxidant function.[36,77]

The antioxidant role of the extramitochondrial one-electron quinone reductases could be potentiated by the reduction of tocopheroxyl radicals by hydroquinones and ubisemiquinones. Kagan et al.[78] have reported that purified cytochrome b_5 reductase can regenerate Trolox, a soluble analogue of α-tocopherol, by reducing its phenoxyl radical in a process requiring NADH and CoQ_0. As described earlier by Stoyanovky et al.,[79] superoxide accounts for part of CoQ-mediated reduction of phenoxyl radicals by one-electron quinone reductases. The role of the cytochrome b_5 reductase in vitamin E regeneration is in accordance with data presented earlier by Constantinescu et al.,[41,73] who reported the participation of the cytochrome b_5 reductase in NADH-driven recycling of α-tocopherol in erythrocyte membranes. Finally, the stabilization of extracellular ascorbate by the transplasma membrane redox system can also contribute to enhancing antioxidant protection both in the aqueous and lipid phases.[55]

A protective role for soluble two-electron quinone reductases against oxidative stress is generally accepted since their reaction mechanism avoids generation of the semiquinone intermediate, and thus prevents superoxide production.[10] In reconstituted systems containing CoQ and NADH, DT-diaphorase inhibits lipid peroxidation initiated by lipophilic azocompound 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), whereas in phospholipid vesicles lacking either NADH or DT-diaphorase,

peroxidation products are observed. Also, treatment of isolated hepatocytes with dicumarol, a potent inhibitor of DT-diaphorase, interferes with the protection provided by CoQ against adriamycin-induced oxidative damage.[12,45] Also, the soluble NADPH-CoQ reductase, which remains insensitive to low concentrations of dicumarol, inhibits AMVN-induced peroxidation of phospholipid liposomes containing CoQ in the presence of NADPH.[47]

The participation of CoQ in the mantenance of the plasma membrane redox status in various physiological processes is indicated by several types of evidence. Serum contains many factors necessary for cells to grow, but also a number of different antioxidants such as ascorbate and CoQ, among others. Upon serum withdrawal, the extracellular redox balance shifts to oxidant and an oxidative stress is induced in cells, which can be prevented by the addition of soluble antioxidants.[19,80,81]

As a consequence of the oxidative stress, serum-deprived cells undergo lipid peroxidation.[17] It is interesting that mitochondria-deficient ρ^o HL-60 cells, which show higher plasma membrane-associated CoQ levels, manifest lower lipid peroxidation when cultured without serum. These changes are reflected in other activities related to plasma membrane such as the ascorbate stabilization and the NADH-AFR oxidoreductase activity. Both activities are stimulated in mitochondrial-deficient ρ^o HL-60 cells. This set of data evidences that ascorbate stabilization and NADH-AFR oxidoreductase are not influenced by mitochondria, but by plasma membrane CoQ.[23] It is also noteworthy that ρ^o HL-60 cells do not show higher levels of lipid peroxidation than wild type HL-60 cells when growing in nonstressing conditions. Thus, the absence of mitochondria does not lead to oxidative stress or in other words, mitochondria may have no role in the protection against nonmitochondrial lipid peroxidation.

Culturing under oxidative stress conditions induces an increase in CoQ content of plasma membranes from both wild type and ρ^o HL-60 cells, but this effect is much more pronounced in the latter cells (Table 5.1). Moreover, parental HL-60 cells loaded with exogenous CoQ mimick the resistance exhibited by ρ^o HL-60 cells in the absence of added CoQ. This effect is also independent of the redox status of the CoQ, which demonstrates the participation of quinone reductase activities that enable oxidized CoQ to perform as $CoQH_2$ does.[17]

TABLE 5.1
Changes in Plasma Membrane CoQ Content and Their Effect on Plasma Membrane Redox Activities

Cell Type	Treatment	CoQ Content vs. Control	Activity Measured	Activity vs. Control (100%)	Ref.
HL-60 cells	Ethidium bromide	140% (Q_{10})	Ascorbate stabilization	152%	[23]
			NADH-AFR oxidoreductase	135%	[23]
ρ^o HL-60 cells	Serum-withdrawal	281% (Q_{10})	Ascorbate stabilization	not tested	[23]
K562	TPA	170%	Ascorbate stabilization	190%	[61]
			NADH-AFR oxidoreductase	150%	[61]
Rat hepatocytes	Tocopherol-depleted diet	138% (Q_9) 162% (Q_{10})	NADH-AFR oxidoreductase	191%	[21]
Yeast cells	Hydrogen peroxide	132% (Q_6)	Ascorbate stabilization	260%	[72]
Yeast cells	CoQ addition	145% (Q_6)	Ascorbate stabilization	124%	[4]

Yeast cells are adapted to grow in the absence of antioxidants, but when exposed to hydrogen peroxide as a source of oxidative stress, they display a rearrangement in CoQ_6 distribution similar to that presented in HL-60 cells. Again, oxidative stress potentiates the ability of yeast cells to stabilize extracellular ascorbate. In parallel to the elevation of CoQ_6 levels at the plasma membrane, endomembranes are significantly depleted in this lipid, indicating that in these short-term experiments, the increase in plasma membrane CoQ is likely the result of an alteration in the distribution pattern of the lipid among membranes, and not of its enhanced biosynthesis (Table 5.1).[72] This observation is consistent with those reported in rats *in vivo* using carbon tetrachloride as a source of oxidative stress.[50] This new distribution of CoQ might enhance the antioxidant capacity of the first membrane barrier against external ROS.

Rodents are very useful *in vivo* models due to their metabolic similarity to humans. The induction of the plasma membrane CoQ-dependent antioxidant system in animals has been demonstrated using a vitamin E-deficient diet. This diet leads to a chronic oxidative stress in Long-Evans rats, which provokes an increase in both the plasma membrane CoQ content, and CoQ-dependent redox enzymes activities such as DT-diaphorase, NADH-AFR oxidoreductase, and other NAD(P)H-dependent oxidoreductases.[21] In these long-term experiments, a net increase in total CoQ_9 and CoQ_{10} due to enhanced biosynthesis is also observed (Table 5.1).[82] The induced plasma membrane CoQ-dependent antioxidant system efficiently prevents the lipid peroxidation in the presence of NAD(P)H. Accumulation of lipid peroxides is also prevented by the activation of phospholipase A_2 activity found in vitamin E-deficient animals.[21]

From all the previously discussed data, a new question arises: how is CoQ selectively accumulated in the plasma membrane under oxidative stress? Although there is no answer to this question yet, this phenomenon suggests either: (i) a CoQ transport from the endomembranes to the plasma membrane, via a specific protein or CoQ-enriched vesicles, or (ii) a redirection of the newly-synthesized CoQ favoring targeting to the plasma membrane. These systems do not exclude each other or complementary ones. The second hypothesis could be related to the up-regulation of CoQ biosynthesis observed after oxidative metabolism stimulation by treatment with thyroid hormone.[2]

The increase in plasma membrane, together with the depletion of mitochondrial CoQ levels, suggests that there may be some relationship or regulation between mitochondrial and extramitochondrial CoQ contents. Regardless of the molecular mechanism, the results obtained in such different eukaryotic systems suggest that the reinforcement of the CoQ-based plasma membrane antioxidant capacity could be a well-established eukaryotic defense system against oxidative damage induced by environmental ROS.

5.6 ROLE OF PLASMA MEMBRANE CoQ IN THE CONTROL OF CELL GROWTH, DIFFERENTIATION, AND APOPTOSIS

Evidence has accumulated for the role of transplasma membrane electron transport in the control of cell growth. Addition of extracellular impermeable oxidants stimulates the growth of cultured cells under serum-limiting conditions.[24,57,83] On the other hand, antiproliferative agents such as adriamycin and retinoic acid[24] or tumor necrosis factor[66] inhibit the plasma membrane redox transport.

The participation of CoQ in the electron transfer across the plasma membrane,[9,51] suggests that this molecule could be also related to growth control. Addition of CoQ_{10} to culture media stimulates cell growth in the absence of serum in several cell lines including HeLa, BALB/3T3, HL-60,[16,17,84] and K562.[22] Inhibitors that compete for CoQ at the plasma membrane redox system, such as capsaicin, prevent cell growth and induce apoptosis in tumor cells.[85–87] The basis of cell growth stimulation by CoQ remains to be fully determined, but it could be explained, at least partially, by the fact that CoQ decreases apoptotic cell death induced by serum deprivation,[17] (see below).

Since serum contains CoQ, part of the mitogenic action of serum has been attributed to CoQ.[16] Growth stimulation by impermeable external oxidants like ferricyanide and by CoQ_{10} are additive, which suggests that both compounds are acting at different levels. Cytometric analysis of cells

grown in the absence of serum, but in the presence of either ferricyanide or CoQ_{10}, show that stimulation by extracellular electron acceptors produces a large increase in G_1 phase protein, whereas CoQ_{10} increases DNA synthesis and transition to G_2 phase.[16]

Plasma membrane redox activity is also modulated by cell differentiation processes. Agents that induce differentiation, such as retinoic acid, have been related in some cases with a decrease of redox activity in plasma membrane.[58] However, differentiation processes induced by TPA in HL-60 cells increase the plasma membrane redox activity, stimulating ferricyanide reductase activity in the first 24 h of differentiation and changing the NAD^+/NADH ratio.[59,60] Also, TPA-induced differentiation of K-562 cells increases ascorbate stabilization by whole cells, NADH-AFR reductase, and CoQ_{10} levels at the plasma membrane.[61]

Cell population is based on the equilibrium among proliferation, differentiation, and cell death. Although antioxidants including CoQ can stimulate cell proliferation in serum-limiting conditions[83,84] (see above), a likely function of antioxidant CoQ in extramitochondrial membranes is the prevention of apoptotic program development, resulting in the maintenance of cell population.[17]

Mild oxidative stress has been related to the development of cell death by apoptosis,[88] and Bcl-2, which is one of the main antiapoptotic proteins, acts through an antioxidative pathway suppressing lipid peroxidation developed after the apoptotic signal.[81] Serum or growth factor withdrawal is a way to initiate apoptosis that courses through an increase of peroxidation levels in membranes.[89,90] Consistent with their ability to reduce levels of lipid peroxidation, addition of antioxidants, including CoQ, to cultures in the absence of serum results in enhanced protection against cell death.[17,19,20,80]

Mitochondria are organelles playing an important role in the control of cell death by releasing apoptogenic factors such as cytochrome *c* and apoptosis-inducing factor, and the dissipation of the electrochemical gradient across the inner membrane.[91] However, disrupting the plasma membrane electron chain with vanilloid inhibitors induces apoptosis through the modification of the redox equilibrium of cytosol before dissipation of the mitochondrial membrane potential is observed.[85,86] Addition of CoQ_{10} to serum-free media also maintains the growth in mitochondria-defective ρ^o cells produced by a long-term treatment with ethidium bromide,[17,71] indicating that this effect is independent of the mitochondrial function of the quinone.

Consistent with a role for the CoQ-dependent redox system in the regulation of the initial events leading to cell death caused by serum withdrawal, the protection afforded by CoQ (or other antioxidants affecting its redox state) is independent of the expression of Bcl-2.[20] If we take into consideration that Bcl-2 protein has not been localized at the plasma membrane,[91] the CoQ-dependent antioxidant system appears to play a crucial role in protecting the plasma membrane from oxidative signals, before the participation of intracellular systems is needed.

Apoptosis induced by serum or growth factor withdrawal courses through the activation of a magnesium-dependent, neutral sphingomyelinase located at the plasma membrane, with a concomitant elevation of ceramide acting as an intracellular secondary messenger, which causes cell cycle arrest or apoptosis.[92,93] Since the cytosolic antioxidant glutathione directly inhibits neutral sphingomyelinase *in vitro*, and glutathione-depleted cells show activation of the enzyme,[94] this is probably a target enzyme for regulation by plasma membrane antioxidants. Addition of CoQ_{10} to serum-free medium inhibits apoptosis and decreases long-term ceramide accumulation in HL-60 cells.[17]

A higher content of CoQ at the plasma membrane might then determine a higher resistance to developing apoptosis after serum withdrawal. This is especially relevant in mitochondria-defective ρ^o cells. Some of these cells, such as the fibroblast-derived ρ^o701.2a cell line, are more sensitive to serum withdrawal than parental cells, and are protected by the overexpression of Bcl-2.[95] However, ρ^o lines derived from HL-60 cells, which show increased endogenous levels of CoQ and elevated electron transport activity at the plasma membrane (Table 5.1),[23] accumulate much less ceramide and are more resistant to serum removal than parental HL-60 cells.[17] Recently, we have found that early events related to the development of the apoptosis program, such as early activation of neutral sphingomyelinase, ceramide accumulation, and capsase activation are also prevented by CoQ_{10} in a process not requiring the expression of Bcl-2.[96]

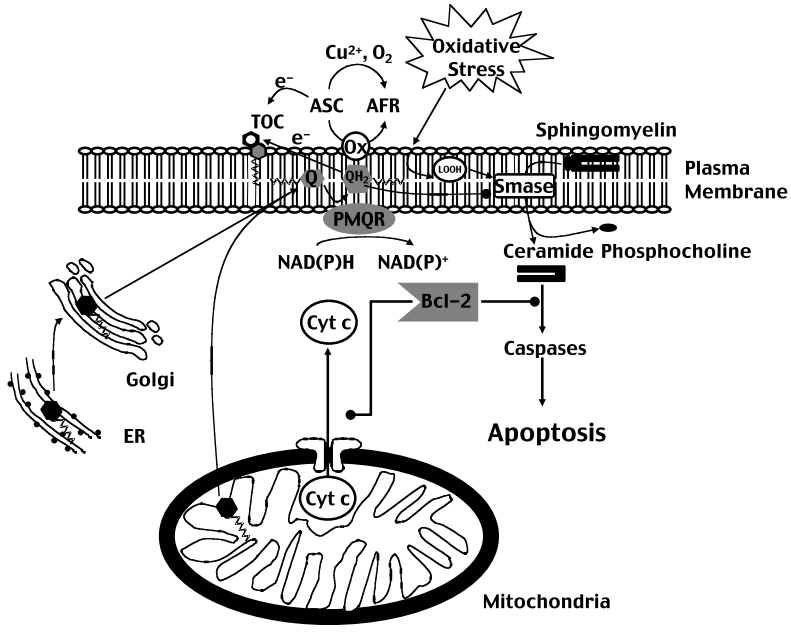

FIGURE 5.2 CoQ regulation of oxidative stress-induced cell death. Oxidative stimuli such as serum withdrawal induce an increase in lipid hydroperoxide (LOOH) levels resulting in activation of a magnesium-dependent, neutral sphingomyelinase (Smase), which hydrolizes sphingomyelin to phosphocholine and ceramide. Ceramide acts as an intracellular secondary messenger activating the caspase cascade, which results in cell death by apoptosis. The antiapoptotic protein Bcl-2 inhibits caspase activation and cytochrome *c* (Cyt *c*) release from mitochondria. CoQ (Q) and plasma membrane CoQ-reductases (PMQR) play protective roles that are independent of Bcl-2 and mitochondria. Increase of plasma membrane CoQ under oxidative stress may be the result of enhanced biosynthesis and/or translocation from intracellular reservoirs such as the endoplasmic reticulum-Golgi system and mitochondria. CoQ-reductase activity at the plasma membrane is also increased as a result of enhanced expression of the cytochrome b_5 reductase and translocation of soluble DT-diaphorase to the plasma membrane. Increasing the levels of the hydroquinone (QH_2) results in lower lipid peroxidation and prevents sphingomyelinase activation. Ascorbate (ASC) stabilization through transmembrane NADH-AFR reductase may also contribute to antioxidant protection via α-tocopherol (α-TOC) regeneration.

This protective system based on extramitochondrial CoQ is in contrast with a role for mitochondrial CoQ in promoting apoptosis. In this case, proapoptotic action of CoQ relies on free radical generation by reduced and semireduced CoQ species, due to an impairment of proper function of the inner membrane electron chain,[97] likely after cytochrome *c* release from mitochondria. The possibility exists that the increase in CoQ associated with the plasma membrane observed under several types of oxidative stress reflects not only enhanced biosynthesis related to the prevailing oxidative status,[2] but also a change in the distribution pattern of CoQ among cellular membranes to avoid prooxidative reactions in intracellular membranes. This very interesting hypothesis is summarized in Figure 5.2 and some aspects are currently under investigation.

5.7 CONCLUSIONS AND PERSPECTIVES

Minor changes in the cellular redox equilibrium can modulate enzyme activities and signal transduction pathways and gene expression, but major changes alter the cell physiology and trigger processes such as growth arrest and apoptosis. Evidence has accumulated supporting that, in addition to the mitochondria, extramitochondrial membranes (mainly the plasma membrane) participate in the maintenance of the cellular redox equilibrium. Analogous to the inner mitochondrial membrane, CoQ appears to be a

central molecule in the extramitochondrial antioxidant machinery. A delicate balance between prooxidant and antioxidant activity of CoQ is maintained by the equilibrium between one- and two-electron quinone reductases, and its relationship with other hydrophilic and lipophilic antioxidants.

A relationship must exist between mitochondrial CoQ and that of the plasma membrane. The study of mechanisms involved in CoQ distribution among cell membranes will help to elucidate the actual contribution of this molecule to the regulation of cellular functions such as stress protection, signal transduction and DNA transcription, differentiation, and cell death.

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6 The Role of Coenzyme Q in Lysosomes

Hans Nohl and Lars Gille

CONTENTS

6.1 INTRODUCTION

The wide distribution of ubiquinones (UQ) in almost all biological systems suggests a major role of this biomolecule in the maintenance of homeostasis.[1] The isoprenic side chain, which is linked to the benzoquinone head group, anchors the molecule in the lipid phase of biological membranes. Nevertheless, UQ is inhomogenously distributed in biomembranes. This observation indicates that loading of the various biomembranes with UQ is actively regulated rather than being a result of passive partitioning. This fact strongly suggests that in some membranes, UQ is more than a building block of the membrane architecture, although UQ is thought to contribute to the structural integrity of biomembranes. The highest values of UQ are found in mitochondria, Golgi vesicles, and lysosomes.[2] The recognition that UQ exerts bioenergetic functions in mitochondria has focused the interest of many scientists for more than four decades and culminated a Nobel prize for Peter Mitchell.

Very little is known about the role of UQ associated with Golgi vesicles. Crane reported that in Golgi membranes, UQ may contribute to proton translocation assuming the existence of adequate redox-couples that are functionally in contact with UQ.[3] It seems to be clear that Golgi apparatus sorts many types of molecules after being transported from their sites of generation to these vesicles. Whether UQ is one of these molecules passing through the Golgi structure or whether it exerts bioenergetic functions as suggested by Crane cannot be answered yet. Even less is known with respect to why lysosomes contain unusually high amounts of UQ. All enzymes present in the lumen of lysosomes are optimally active near the pH of 5 maintained within these organelles. The fact that lysosomal enzymes require an acid pH for optimal activity protects the cytoplasm against damage should leakage occur. The lysosomal membrane is thought to contain a transporter protein that utilizes the energy of ATP hydrolysis to pump protons into the lumen of lysosomes.[4] However, it is not clear whether this proton translocator works *in vivo* since ATP generated elsewhere is not likely to serve as a reliable energy source. In contrast, UQ in analogy to its function in the mitochondrial inner membrane, can be considered as a reliable proton shuttle that can interact with adequate reductants and oxidants. Following this hypothetical concept we systematically studied the existence of lysosomal redox-couples that could interact with ubiquinones, and the pathway of protons associated with redox-cycling of this freely movable membrane component.

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6.2 RESULTS

The diversity of shapes and sizes of lysosomes contrasts with the rather uniform ultrastructure of all other cellular organelles. This heterogeneity requires special techniques to prevent contamination with other organelles or fragments of organelles. This problem is often overcome by preloading lysosomes with Triton WR-1339 or dextran to increase their sedimentation constant and to adjust the different sizes. However, loading of lysosomes with these foreign compounds was reported to stimulate autophagy. We, therefore, isolated native lysosomes from liver homogenates of rats by isopycnic centrifugation through a self-generating iodixanol gradient (ultracentrifugation of a light mitochondrial fraction in a gradient of 17.5% iodixanol for 3 h 40 min at 230,000 × *g*).[5] Identification of the lysosomal fraction was made safe by the determination of the presence of acid phosphatase, which is a characteristic marker enzyme of lysosomes.[6] The possible contamination with microsomal and mitochondrial fragments was assessed from the typical marker enzymes of these organelles (CN^- insensitive NADH-cytochrome *c* reductase for microsomes, succinate dehydrogenase for mitochondria).[7,8] By these criteria we selected lysosomal fractions for our studies that contained neglectable amounts of fragmented mitochondria and microsomes.

Ubiquinone contents in lysosomes were determined by means of HPLC in combination with UV detection at 275 nm following extraction from lysosomal membranes with hexane.[2] Ubiquinone and ubiquinol standards were used for the assessment of the reduction pattern. The analysis revealed that 70% of the total UQ was in fully reduced form. The presence of totally reduced UQ in addition to lower amounts of oxidized UQ suggested the existence of redox-couples in lysosomes supplying reducing equivalents to UQ. Addition of NADH to lysosomal suspensions resulted in the appearance of ubisemiquinone radicals (Figure 6.1B) and in further increase of divalently reduced UQ (Figure 6.1A). The flow of reducing equivalents from the external NADH to UQ, therefore, occurs in accordance with the chemistry of UQ by two consecutive one-electron reduction steps. Shuttles that can act between NADH providing a pair of electrons and UQ, which accepts single electrons only are iron-coordinating proteins such as FeS proteins or heme iron. Accordingly, a *b*-type cytochrome was identified that was found to undergo reduction when NADH was added (Figure 6.2). Soret bands obtained from redox-difference spectra revealed the identity of this one-electron carrier (Figure 6.2). NADH was oxidized immediately after being in contact with lysosomes. This suggests the existence of an adequate catalyst. FAD and FMN are prosthetic groups of dehydrogenases also expected to be involved in lysosomal oxidation of NADH. HPLC analysis revealed the existence of both cofactors. Based on the fivefold higher presence of FAD, however, the latter seems to be more important than FMN. This was further supported by the kinetic similarities of NADH-induced FAD and cytochrome *b* reduction (Figure 6.3). The concerted response to lysosomal NADH oxidation suggests the involvement of FAD in cytochrome *b* reduction. Steady state levels of reduced cytochrome *b* were found to depend on the availability of UQ in lysosomes. Removal of UQ from lysosomal membranes had a stabilizing effect on the reduction state of cytochrome *b* while reincorporation accelerated reoxidation (Figure 6.4). In agreement with the redox-chemistry, which demands a particular order of redox-couples, the oxidant effect of ubiquinone on cytochrome *b* reveals that single electrons for ubiquinone reduction are provided by cytochrome *b*. Disappearance of NADH in contact with lysosomes was accompanied by the consumption of oxygen (Figure 6.5). The chemistry of oxygen reduction suggested the formation of superoxide radicals as the first reduction product. The expected reaction product was analyzed by means of ESR spectroscopy in the presence of DMPO for spin trapping. However, the spin adduct observed revealed the presence of trivalently vs. univalently reduced dioxygen (Figure 6.6). The quartet ESR signal was clearly derived from spin trapping of free $HO^{\bullet}$ radicals and not from the molecular derangement of an original DMPO/$^{\bullet}OOH$ adduct. This was concluded from the insensitivity of the ESR signal to SOD. The lack of any effect of SOD together with the absence of $O_2^{-\bullet}$ spin adducts does, however, not exclude the possibility that $O_2^{-\bullet}$ radicals were formed. Superoxide radicals may

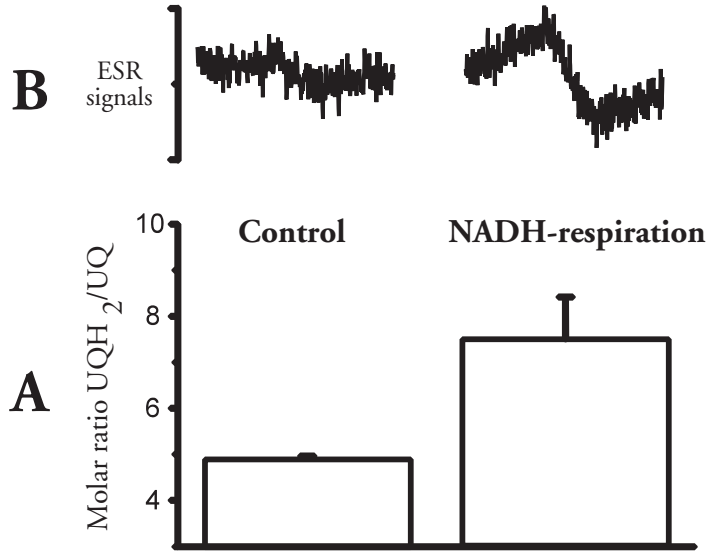

FIGURE 6.1 Shift of the redox-state of lysosomal ubiquinone induced by NADH in the presence of rotenone to exclude the involvement of mitochondrial ubiquinones. (A) In NADH-respiring lysosomes the oxidation state of ubiquinone is shifted in favor of ubiquinol (UQH_2) under anaerobic conditions. Control experiments were without NADH. Conditions: 50 μl lysosomal suspension were mixed with appropriate substrates and inhibitors giving a final volume of 250 μl. This mixture was kept under argon for 30 min at 37°C. After extraction with an organic solvent, UQ and UQH_2 contents were determined from RP-HPLC with UV-detection at 275 nm ($n = 3$; error bars correspond to s.d.). (B) ESR signal induced in lysosomal fractions following addition of NADH. The signal exhibits spectral properties similar to ubisemiquinones in mitochondria. In control experiments without NADH the signal was not observed. Conditions: 100 μl lysosomal suspension was mixed with substrates and inhibitors giving a final volume of 200 μl. After 5 min of incubation, the sample was placed into liquid nitrogen. The ESR measurements were performed at 200 K. Spectrometer settings: microwave frequency 9.47 GHz, center field 3380 G, sweep 100 G, modulation amplitude 4 G, receiver gain 1 E6, scan rate 35 G/min, time constant 0.163 s, scans 3.

readily undergo accelerated spontaneous dismutation when generated in a condensed form close to the catalyst, which promotes homolytic cleavage of the $O_2^{-\bullet}$ dismutation product H_2O_2. The existence of redox-cycling UQ in lysosomal membranes raises the question as to whether protonation/deprotonation steps following the uptake and release of electrons is side-directed. Considering the more acidic pH in the lumen of the active lysosomes, we followed this question by using the uncharged spin probe Tempamine, which readily diffuses across the lysosomal membrane being arrested once inside the lumen, by the addition of a proton.

The more protons are accumulated in electron transferring lysosomes, the more the paramagnetic spin probe will be arrested inside, thereby increasing the characteristic ESR signal with respect to the controls (Figure 6.7). Contribution of spin probes outside the lumen of lysosomes to the overall signal intensity was prevented by extinguishing this fraction after the addition of ferricyanide. This highly sensitive method revealed that proton translocation occurred when NADH was present to run the lysosomal redox-chain. For an evaluation of the significance of this NADH-related proton transporter we also measured ATP-dependent proton accumulation. Both systems were equally active. Proton translocation initiated by the addition of NADH was, however, dependent on the availability of oxygen (Figure 6.7). This observation reveals that oxygen most probably accepts electrons at the oxidant site of ubiquinone, thereby keeping linear electron transfer associated with unilateral proton transfer running.

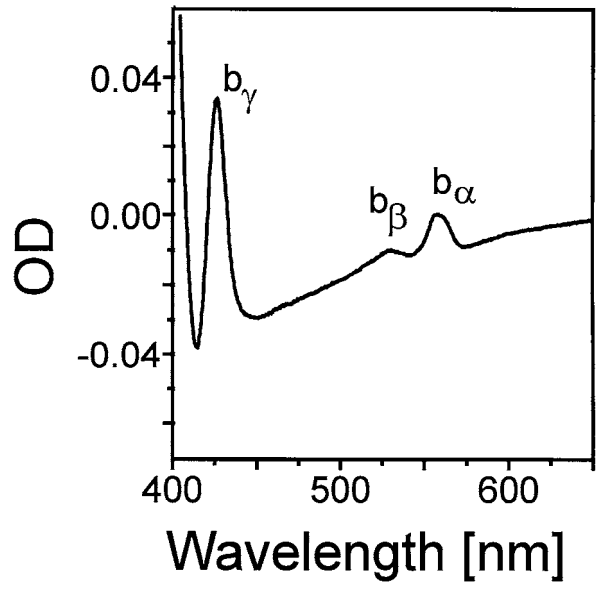

FIGURE 6.2 Redox-difference spectrum of the lysosomal fraction obtained after the addition of NADH. The spectrum exhibits strong absorbtion peaks at 559 nm (α-band), 427 nm (γ-band), and a weak absorption between 520 and 530 nm (β-band) suggesting the presence of *b*-type cytochromes different from cytochromes in mitochondria and microsomes. Conditions: 1 mg protein of the lysosomal fraction was dissolved in 1ml preparation buffer and spectra were recorded prior to and after the addition of NADH (0.1 mM final concentration). The redox-difference spectrum was calculated by substraction of both spectra.

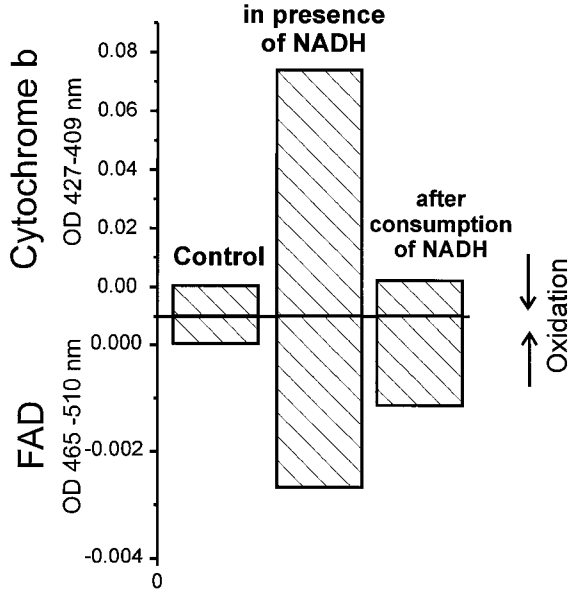

FIGURE 6.3 Concerted reduction of FAD and *b*-type cytochromes in lysosomes upon the addition of NADH. The reduction state of *b*-type cytochromes and FAD was obtained from absorption differences at 427 nm–409 nm and 465 nm–510 nm, respectively. NADH induced the simultaneous reduction of both electron carriers. After the total consumption of NADH reoxidation of both electron carriers was observed. Conditions: The kinetics of cytochrome *b* and FAD reduction/oxidation was followed in a dual wavelength spectrophotometer using 0.8 mg of lysosomal protein equilibrated with 5 nmol NADH in the air-saturated preparation buffer.

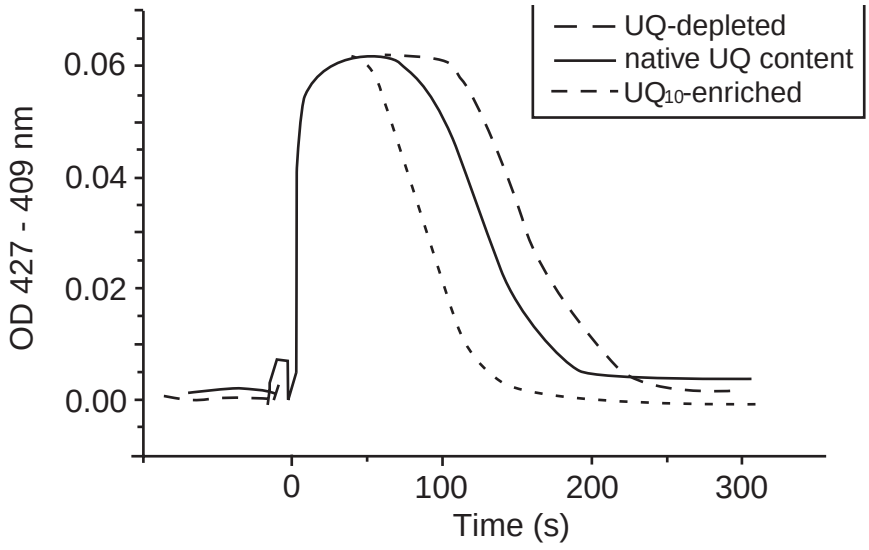

FIGURE 6.4 The rate of cytochrome *b* reoxidation depends on the availability of oxidized ubiquinones in lysosomes. Lyophilized lysosomes were extracted with heptane in order to remove UQ from the organelles. These lysosomes were reconstituted with different amounts of UQ; (dashed line) no UQ; (solid line) native amount of UQ; (dotted line) excess of UQ_{10}. After rehydratization, equimolar amounts of NADH were applied and cytochrome *b* reduction/oxidation kinetics were followed photometrically. NADH-induced cytochrome *b* reduction was a function of the amount of UQ present in the lysosomal membrane. Conditions: Lyophilized lysosomes were treated with heptane in order to extract ubiquinone.[14] Each 20 mg lysophilisate was reconstituted (A) with hexane only, (B) 3 nmol UQ in hexane, and (C) 100 nmol UQ_{10} in hexane. After removal of organic solvents, the dry lyophilisate was resuspended in water yielding an organelle suspension with a protein concentration of about 25 mg/ml. The reduction/oxidation kinetics were measured as described in Figure 6.3.

6.3 DISCUSSION

Lysosomes are organelles specialized for subcellular digestion. They contain a wide variety of different acid hydrolases that all require an acid pH milieu for optimal activity. Molecules that penetrate into the lumen must be uncharged to overcome the lipid barrier. Once present in the lumen, they become charged by picking up a proton in the acidic environment.[9] Therefore, these molecules enter the lysosomes more rapidly, then they leave and become highly concentrated inside. It is clear that this concerted action between substrate accumulation and optimal enzyme activity requires a permanent supply of protons from the extramembraneous space. Our finding that over 70% of UQ was in the divalently reduced state was taken as a strong indication of the redox-function of lysosomal UQ. Redox-cycling of UQ is accompanied by the stepwise addition and release of protons. The intermediates involved differ in their polarities, which allows them to move from the more lipophilic phase of the membrane to the polar head group region or vice versa. In mitochondria these peculiarities of redox-cycling ubiquinones contribute to unilateral proton translocation exclusively driven by the flux of electrons.

In lysosomal membranes, the flux of reducing equivalents to and from ubiquinone exists as well. Reducing equivalents are provided from cytosolic NADH, a substrate that is present in abundance. We have demonstrated that oxygen is required as the terminal electron acceptor to run this coupled redox-chain. In the absence of oxygen, proton accumulation was clearly reduced strongly suggesting that redox-cycling ubiquinones were involved in proton translocation. From UQ extraction/reincorporation experiments, cytochrome *b* can be excluded as the reductant of oxygen whereas in contrast, ubiquinones are required as oxidants for cytochrome *b*. The latter accepts the reducing equivalents

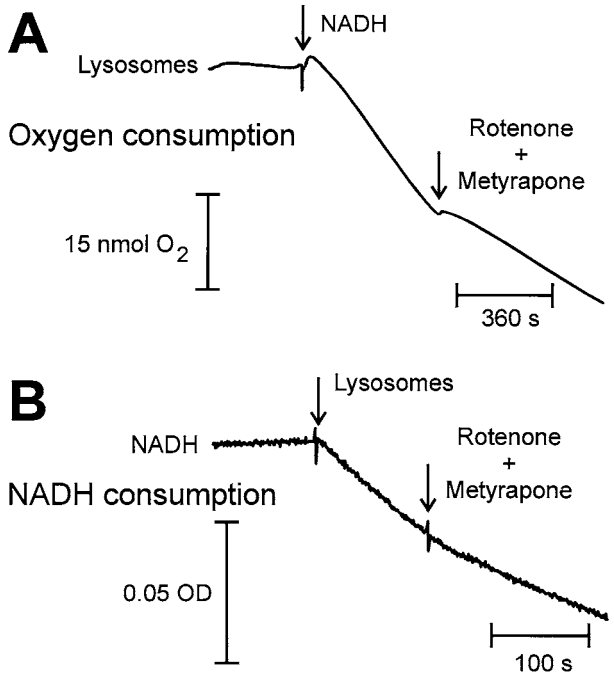

FIGURE 6.5 Neither mitochondrial nor microsomal inhibitors of electron transfer reactions prevented the NADH-dependent oxygen consumption of the lysosomal fraction, suggesting redox-processes are native functions of lysosomal membranes. (A) Oxygen consumption of lysosomal fractions followed with a Clark-type electrode was started by the addition of NADH. The presence of rotenone (prevention of mitochondrial NADH oxidation) and metyrapone (prevention of microsomal NADH oxidation) decreased oxygen consumption by 50%. (B) NADH consumption was measured photometrically at 340 nm–400 nm. Inhibition of NADH-consumption in the presence of the above inhibitors reflected the decrease of oxygen uptake. A further increase of inhibitor concentrations did not reduce the residual rates of NADH and oxygen consumption. Conditions: (A) 4 mg protein was placed in the reaction vessel (560 μl) of a Clark-type electrode and the oxygen consumption was recorded after addition of NADH (3.6 mM final concentration). The inhibitors rotenone (10 μg/ml) and metyrapone (2 mM) were present in order to eliminate the contribution of mitochondria and microsomes, respectively. (B) 0.8 mg lysosomal protein in 1ml buffer was supplemented with NADH (50 μM final concentration). The decay of the NADH absorption was followed photometrically at 340 nm minus 400 nm ($\epsilon_{340-400}$ = 6290 mol*l^{-1}*cm^{-1}).[15] Inhibitors were applied as described above.

from FAD, which indicates the presence of a lysosomal NADH dehydrogenase. Evidence for the existence of this initial redox-couple of the lysosomal electron transfer chain comes from kinetic similarities of FAD and cytochrome *b* reduction. We have repeatedly shown that redox-cycling $UQ^{\bullet -}$ may undergo autoxidation.[10,11] The detection of DMPO/$^{\bullet}$OH spin adducts suggests that UQ, which accepts two single electrons from cytochrome *b*, undergoes autoxidation in the semireduced state. Superoxide radicals emerging from autoxidation drive the reaction by subsequent dismutation. The lack of direct $O_2^{\bullet -}$ detection suggests the existence of this autoxidation product close to the acid pH milieu of the lumen, which accelerates spontaneous dismutation. H_2O_2 formed can also drive electron flux through the lysosomal redox-chain by using the odd electron of ubisemiquinone for reductive homolytic cleavage.[12] The resulting $HO^{\bullet}$ radical was captured by spin trapping with DMPO. We have recently shown that ubisemiquinones undergoing autoxidation must release their protons.[13] The pK of the $UQH^{\bullet}$ radical favors deprotonation in the alkaline pH, which means that in the physiological pH range, anionic ubisemiquinones transfer their electrons to oxygen while the proton is translocated into the lumen. Uncharged ubisemiquinones are ready to accept a second electron giving rise to

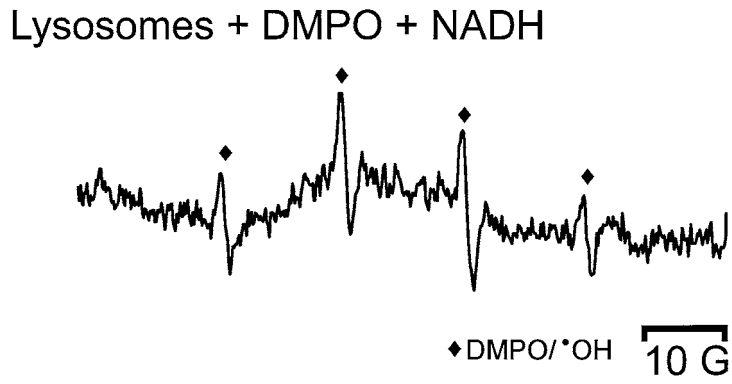

FIGURE 6.6 NADH respiration of lysosomes results in the trivalent reduction of oxygen giving rise to the formation of HO• radicals, which were detected by ESR spin trapping with DMPO. The ESR spectrum exhibits the typical ESR splitting characteristics of a DMPO/•OH addcut. In the absence of NADH, no ESR signal was detected. Rotenone was present in the experiment in order to exclude radical formation from possibly contaminating mitochondria. Conditions: 5 mg of lysosomal protein was supplemented with DMPO (118 mM final concentration), NADH (4 mM final concentration), DTPA (2 mM final concentration) in presence of rotenone (20 µg/ml final concentration) giving a final volume of 500 μl. The latter was transferred into a ESR quartz flat cell and the ESR measurements were performed 2 min after starting the reaction. Spectrometer settings: microwave frequency 9.81 GHz, center field 3495 G, sweep 80 G, modulation amplitude 1 G, receiver gain 1×10^6, scan rate 114 G/min, time constant 40.96 ms, scans 1, temperature 298 K. Spin adducts were identified according to Buettner.[16]

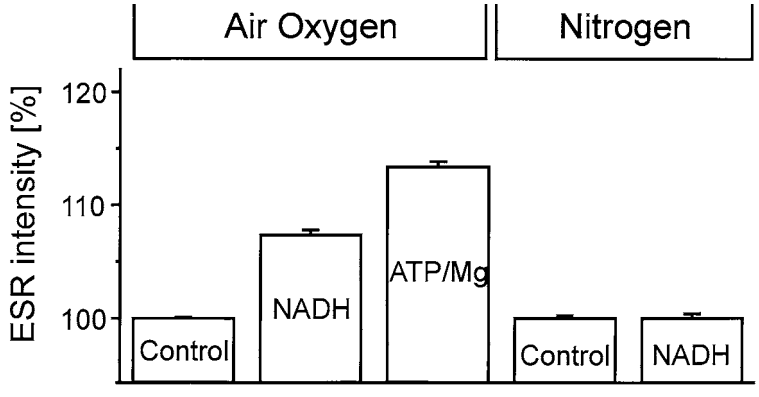

FIGURE 6.7 Intralysosomal accumulation of the spin probe Tempamine following protonation driven by NADH respiration. Tempamine was used as a paramagnetic spin probe to detect a proton accumulation in lysosomes after being arrested by protonation. The ESR signal of the spin label remaining in the exterior was quenched by ferricyanide. Under aerobic conditions, NADH as well as ATP/Mg caused an accumulation of Tempamine in lysosomes in comparison to the control. However, under anaerobic conditions, this effect of NADH was not observed. Conditions: 25 μl of a lysosomal suspension were mixed with 5 μl Tempamine stock solution and 5 μl preparation buffer or NADH stock solution. After incubation, 5 μl $K_3[Fe(CN)_6]$ stock solution was added giving the following final concentrations of: 27.5 mg/l protein, 1 mM Tempamine, 200 mM $K_3[Fe(CN)_6]$, and 1.25 mM NADH, if required. The total volume of 40 μl was measured in gas-permeable TFE-tubes using an ESR spectrometer with a dielectric resonator. Measurements were performed in air oxygen or nitrogen atmosphere. Spectrometer settings: microwave frequency 9.71 GHz, center field 3453 G, sweep 60 G, modulation amplitude 1 G, receiver gain 5×10^4, scan rate 21 G/min, time constant 0.163 s, scans 1.

the existence of UQH_2. It can be speculated that steady state formation of ubiquinol from the uncharged fraction of $UQH^{\bullet}$ species protects the lysosomal membrane from oxidative stress established from the compulsory existence of $HO^{\bullet}$ radicals. Although the inevitable production of trivalently reduced dioxygen was shown to keep UQ-related proton translocation running, further studies are required to understand the role of these strong prooxidants. We have also shown that ATP-dependent proton accumulation exists in addition to proton translocation through redox-cycling UQ. The significance of this proton pump is a function of the availability of ATP from mitochondria.[4] In contrast, the relatively high fraction of reduced UQ in lysosomes, even after the complex isolation procedure, indicates that UQ-related proton translocation is not substrate limited.

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Section 1C

Antioxidant Mechanisms

7 Antioxidant Dynamics of Coenzyme Q in Membranes

Etsuo Niki

CONTENTS

7.1 INTRODUCTION

The activity of radical-scavenging antioxidants is determined by several factors including (1) reactivity and stoichiometry toward radicals, that is, how rapidly and how many radicals can be scavenged by the antioxidant, (2) concentration of the antioxidant, (3) fate of antioxidant-derived radical formed when the antioxidant scavenges the radical, (4) location of the antioxidant, (5) mobility of the antioxidant in the microenvironment, and (6) interaction with other antioxidants. The activities of various compounds as antioxidants have been measured both *in vitro* and *in vivo*. *In vitro* activities have been extensively assessed by different methods in various media. It must be well appreciated that the total antioxidant activities are determined, not simply by the reactivities toward radicals, but also by many other factors as described above. In other words, it must be clearly understood which factor is being measured in the *in vitro* experimental system employed, and that it does not always give the antioxidant's total activity *in vitro* or *in vivo*. It is not difficult to measure the reactivity, toward radicals under specific *in vitro* conditions, but it is difficult to assess the antioxidants efficacy *in vitro*.

It has been well documented since the early report of Mellors and Tappel[1] that ubiquinol (UQH_2), a reduced form of coenzyme Q, acts as an antioxidant against lipid peroxidation [2 and references cited therein]. One of the characteristics in the action of ubiquinol as an antioxidant is that it undergoes autoxidation rapidly, which results in a rapid consumption of ubiquinol, formation of hydroperoxyl radical and/or superoxide, and impaired antioxidant efficacy. The ubisemiquinone radical ($UQH^{\bullet}$) may undergo several reactions such as a reaction with oxygen to give ubiquinone (UQ) and hydroperoxyl radical, reduction of α-tocopheroxyl radical to regenerate α-tocopherol (vitamin E), and disproportionation with another ubisemiquinone radical to give ubiquinol and ubiquinone. It has also been reported that the ubisemiquinone radical is capable of decomposing hydrogen peroxide and hydroperoxide to give hydroxyl and alkoxyl radicals, respectively.[3] Some of them are effective for antioxidation, but others are not. The total antioxidant efficacy of ubiquinol is dependent on the relative importance of these reactions.

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7.2 REACTIVITY TOWARD RADICAL AND ANTIOXIDANT ACTIVITY OF UBIQUINOL

The reactivity toward radicals, as determined by chemical structure, is apparently the important factor in determining the antioxidant activity of the compound. The reactivity of ubiquinol toward radicals has been measured by several groups. Mellors and Tappel[1] observed that ubiquinol-6 was as reactive as α-tocopherol toward diphenyl-p-picrylhydrazyl, while Naumov and Khrapova[4] reported that the rate constant for the reaction of ubiquinol with peroxyl radical was smaller than that of α-tocopherol. Mukai and colleagues[5] have measured the rate constant for the reaction of ubiquinol-10 and 2,6-di-tert-butyl-4-(4-methoxyphenyl)phenoxyl radical. Tsuchiya et al.[6] have measured the relative reactivities of ubiquinol-10 and α-tocopherol toward peroxyl radical in the phosphatidylcholine liposomal membranes and found that α-tocopherol was more reactive that ubiquinol-10 by a factor of 4.8. A higher reactivity of α-tocopherol than ubiquinol toward phenoxyl[7] and peroxyl[8] radicals has been also reported by Foti et al. and Barclary et al., respectively.

We have recently measured the reactivities of ubiquinol toward galvinoxyl and peroxyl radicals and compared them with those of α-tocopheryl hydroquinone (TQH_2) and α-tocopherol (TOH).[2,47,48] It was found that ubiquinol was 2.5 and 1.9 times more reactive than α-tocopherol toward phenoxyl and peroxyl radicals, respectively, at 25°C in ethanol, and that it was capable of donating two hydrogen atoms to oxygen radicals.[2] Ubiquinone did not exert appreciable reactivity toward either phenoxyl and peroxyl radicals.

It has been observed that the apparent antioxidant activity of ubiquinol is smaller than that of α-tocopherol against lipid peroxidation in organic solution as judged from either the rate of oxidation or the duration of the inhibition period (or lag phase). The example of the inhibition of oxidation is shown in Figure 7.1 and the relevant data are summarized in Table 7.1 with those for α-tocopherol and α-tocopheryl hydroquinone. These data clearly show that the antioxidant efficacy is determined not only by the reactivity toward the radical, but also by the fate of antioxidant-derived radical. Chemically, α-tocopherol hydroquinone has the highest reactivity toward radicals, but the apparent antioxidant efficacy is the lowest among the three antioxidants: the order in the reactivity toward radicals, inhibition period, and the rate of inhibited oxidation is $TQH_2 > UQH_2 > TOH$, $TQH_2 < UQH_2 < TOH$, and $TQH_2 > UQH_2 > TOH$, respectively. Thus, the relative reactivity toward radical and antioxidant activity are exactly reversed.

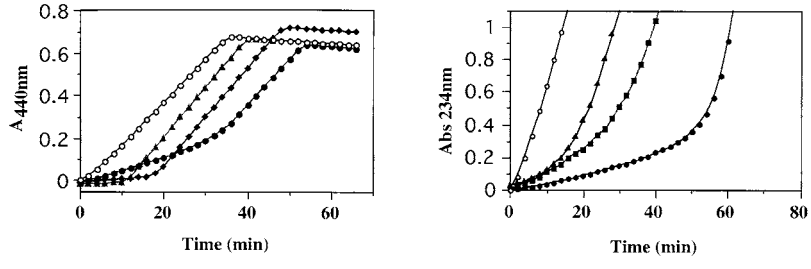

FIGURE 7.1 (A) Effect of ubiquinol (■), α-tocopherol (●), and α-tocopheryl hydroquinone (▲) on the formation of N,N'-diphenyl-p-benzoquinone diimine (DPBQ, λmax = 440 nm) from N,N'-diphenyl-p-phenylenediamine (DPPD). DPPD was incubated in acetonitrile at 37°C with a radical initiatior 2,2´-azobis (2,4-dimethylvaleronitrile) (AMVN) in the absence (○) and presence of antioxidant. (B) Inhibition of oxidation of metyl linoleate by antioxidant. Methyl linoleate (25 mM) was oxidized with AMVN (0.8 mM) in the absence (○) and presence of antioxidant (5 μM) at 37°C in acetonitrile and the accumulation of methyl linoleate hydroperoxide was followed. ○: without antioxidant; ■: ubiquinol; ●: α-tocopherol; ▲: α-tocopheryl hydroquinone.

TABLE 7.1
Activities of Ubiquinol-10 (UQH_2), α-Tocopherol (TOH), and α-Tocopheryl Hydroquinone (TQH_2) as Antioxidant[2,47]

	UQH_2	TOH	TQH_2
Rate constant for reaction with galvinoxyl, at 25°C in ethanol ($M^{-1}s^{-1}$)	6×10^3	2.4×10^3	1.0×10^4
Stoichiometry for reaction with galvinoxyl	2.0	1.0	1.9
Relative reactivities toward peroxyl radical	(1.9)	(1.0)	(6.0)
Inhibition of oxidation of methyl linoleate[a]			
Inhibition period (min)	65	78	13
Stoichiometric number	1.5	1.8	0.30
Rate of oxidation (nM/s)	4.3	1.3	7.6

[a] Methyl linoleate was oxidized at 37°C in acetonitrile under air in the presence of 5 μM antioxidant and 0.50 mM radical initiator, 2,2′-azobis(2,4-dimethylvaleronitrile).

Such a discrepancy in reactivity and activity must arise from the autoxidation of hydroquinone (QH_2) mediated by oxygen and hydroperoxyl radical.

$$X^{\bullet} + QH_2 \rightarrow XH + QH^{\bullet} \tag{7.1}$$

$$\left.\begin{aligned} QH^{\bullet} + O_2 &\rightarrow Q + HO_2^{\bullet} \\ HO_2^{\bullet} + QH_2 &\rightarrow H_2O_2 + QH^{\bullet} \end{aligned}\right\} \text{ n-cycle} \tag{7.2, 7.3}$$

$$QH^{\bullet} + X^{\bullet} \rightarrow Q + XH \tag{7.4}$$

The overall reaction is (7.1 + 7.2 + 7.3 + 7.4)

$$2X^{\bullet} + nO_2 + (n+1)QH_2 \rightarrow 2XH + (n+1)Q + nH_2O_2 \tag{7.5}$$

and the apparent stoichiometric number is $2/(n+1)$, that is, it is 2 and 0.5 when n is 0 and 3, respectively.

It has been proposed in the oxidation of LDL that the hydroperoxyl radical formed in reaction 7.2 is exported into the aqueous phase-out of LDL particles.[9] This is attractive, since under such conditions neither autoxidation of ubiquinol nor prooxidant action by the hydroperoxyl radical should take place. However, it has not been proved experimentally. Superoxide has been detected in the aqueous phase by use of a chemiluminescence probe, 2-methyl-6-phenyl-3,7-dihydroimidazo[1.2-a]pyrazin-3-one (CLA), during the oxidation of α-tocopheryl hydroquinone in micelles, but not for ubiquinol.[47] Apparently, the efficacy of exporting hydroperoxyl radical into the aqueous phase depends on the concentrations of substrates and antioxidants. The pKa of hydroperoxyl radical is 4.8, but it should be present predominantly as hydroperoxyl radical within LDL particles and as superoxide in the aqueous phase.

The antioxidant action of ubiquinol (UQH_2) in the membranes and lipoprotein may be expressed as shown in Figure 7.2. Ubiquinol scavenges the chain-carrying lipid peroxyl radical to give lipid hydroperoxide and ubisemiquinone radical ($UQH^{\bullet}$) (reaction 6), which can undergo several reactions. It may scavenge another peroxyl radical to give hydroperoxide and ubiquinone (UQ) (reaction 7). In this case, the stoichiometric number is 2. It may react with oxygen to give ubiquinone and hydroperoxyl

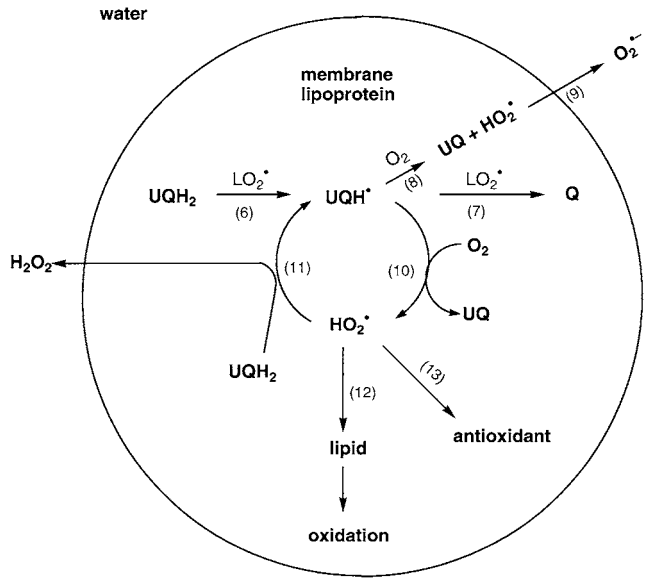

FIGURE 7.2 Proposed scheme for the action of ubiquinol (UQH_2) in the membranes and lipoproteins (see text).

radical (reaction 8), which is exported into the aqueous phase as superoxide (reaction 9). The apparent stoichiometric number is 1. If, on the other hand, the hydroperoxyl radical attacks ubiquinol and induces its autoxidation by a sequence of reactions 10 and 11, then the apparent stoichiometric number becomes less than 1 and the induction period becomes shorter. When the hydroperoxyl radical attacks lipids or proteins to induce their oxidation (reaction 12), ubiquinol serves merely as a chain transfer and does not exert any antioxidant effect. The hydroperoxyl radical may be scavenged by other antioxidants such as vitamin E (reaction 13). Ubisemiquinone may also react with hydrogen peroxide or hydroperoxide, as proposed by Nohl and his colleagues,[3] to give hydroxyl or alkoxyl radicals, which attack substrates (not shown in Figure 7.2). Thus, the total antioxidant potency depends very much on the fate of the semiubiquinone radical.

7.3 ANTIOXIDANT ACTION IN THE MEMBRANES

There are other factors that have to be taken into consideration with respect to the antioxidant action in the membranes and lipoproteins in heterogeneous aqueous dispersions. Coenzyme Q is not only present in the inner mitochondrial membrane, but in lipoproteins, plasma membranes, and all intracellular membranes and it is present largely in the reduced state.[10]

The antioxidant potency of a lipophilic antioxidant against lipid peroxidation in the membranes and lipoproteins is dependent not only on the chemical factors mentioned above, but also on physical factors such as local concentration and mobility within and between the membranes and lipoproteins. It has been shown, for example, that the efficacy of scavenging radicals in the membrane by α-tocopherol decreases as the radical goes deeper into the interior of the membranes[11,12] and the apparent antioxidant activity decreases accordingly.[13,14] α-Tocopherol is incorporated into the membranes in such a way that the active phenolic hydrogen is located near the surface of the membrane, which makes it capable of scavenging radicals attacking from the aqueous phase easily, but makes it less efficient in scavenging radicals within the membrane. Thus, for example, although α-tocopherol is more reactive than β-carotene, β-carotene scavenges the radicals generated in the dimyristoyl phosphatidylcholine liposomal membranes faster than α-tocopherol.[15] The partition and mobility of coenzyme Q in the membranes have been reviewed recently by Kagan, Nohl, and Quinn.[16]

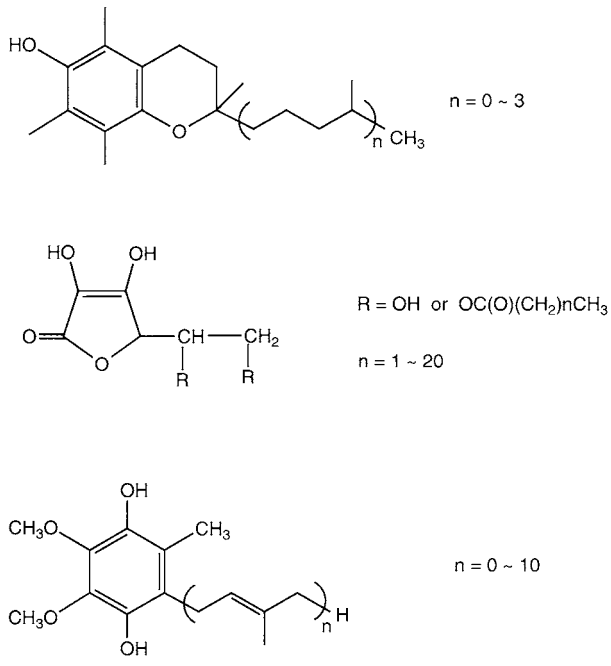

FIGURE 7.3 Analogues of α-tocopherol, ascorbic acid, and ubiquinol with side chain of different length.

The importance of such physical effects of membranes on antioxidant activity is well understood through the action of homologues with different side chains. The effect of side chains on antioxidant activities has been studied for the analogues of vitamin E,[12,14,17–21] vitamin C,[22] and coenzyme Q[23,24] (Figure 7.3). In general, it has been observed that the side chain has little effect on reactivity and antioxidant activity in the homogeneous solution, whereas antioxidant efficacy decreases against lipid peroxidation in the membranes with an increasing number and length of side chains. Kagan et al.[24] measured the efficiency of ubiquinols of varying isoprenoid side chain length in preventing lipid peroxidation induced by iron in microsomes, mitochondria, and synaptosomes and found that the ubiquinols with short isoprenoid chains are much more potent inhibitors of membrane lipid peroxidation than the longer chain homologues. This may be due to a decreased mobility of ubiquinols in the membrane with increasing side chain length. Interestingly, they also found that ubiquinol-0 without a side chain exerted little antioxidant capacity, suggesting the importance of incorporation of the antioxidant into the membrane. Similarly, it is well known that vitamin C in the aqueous phase, although a potent radical-scavenging antioxidant, is not capable of inhibiting lipid peroxidation within membranes.

The side chain affects intermembrane as well as intramembrane mobility. The effect of ubiquinol-1 and ubiquinol-10 on the inhibition of lipid peroxidation in liposomal membranes has been studied.[48] The initiating radicals were generated within the membranes and ubiquinol was incorporated either in the same membranes or in different membranes. As shown in Figure 7.4, ubiquinol-10 suppressed the lipid peroxidation in the membranes in which it was incorporated, but it exerted only poor antioxidant activity against lipid peroxidation taking place in different membranes. On the other hand, ubiquinol-1 inhibited lipid peroxidation efficiently even if it was incorporated into different membranes.

These results suggest that the side chain of ubiquinol, although it is required for incorporation and retention in the membranes, reduces both inter- and intramembrane mobility. Similar effects have been observed for vitamin E against lipid peroxidation in the membranes[17] and oxidative homolysis of erythrocytes.[19]

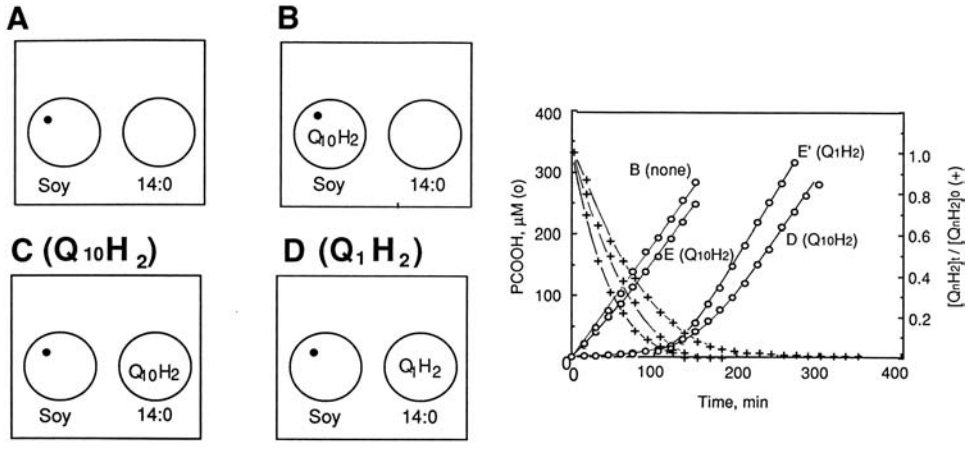

FIGURE 7.4 Effects of location of ubiquinol on the inhibition of oxidation of soybean PC liposomal membranes. Soybean PC (5.1 mM) multilamellar liposomal membranes containing AMVN (1.0 mM) were incubated at 37°C in air in the absence and presence of antioxidant (10 μM) located in different places and the formation of PC hydroperoxides was followed with an HPLC. Dimyristoyl PC (5.9 mM) multilamellar liposomes were also incubated together. A: without antioxidant; B: with ubiquinol-10 incorporated into soybean PC liposomes; C: with ubiquinol-10 incorporated into dimyristoyl PC liposomes; D: with ubiquinol-1 incorporated into dimyristoyl PC liposomes.

7.4 INTERACTION WITH OTHER ANTIOXIDANTS

The radical-scavenging antioxidants function not only individually but also cooperatively and sometimes synergistically with other antioxidants. The most well-documented interaction is the one between vitamin C and vitamin E.[25] Vitamin C present in the aqueous phase, efficiently reduces the vitamin E radical located within the membranes and lipoproteins to regenerate vitamin E and to inhibit, if any, the chain initiation induced by the vitamin E radical.

It has been found that ubiquinol reduces the vitamin E radical,[26–28] which is reasonable since one-electron redox potential for ubiquinol and α-tocopherol is $E_{7.0}$ ($UQH_2/UQH^{\bullet}$) = 0.11[29] ~ 0.24 V,[30] and $E_{7.0}$ ($TOH/TO^{\bullet}$) = 0.48 V, respectively.[31] A substantial deuterium kinetic-isotope effect has been observed in the hydrogen atom transfer reaction between ubiquinol-10 and 5,7-diisopropyltocopheroxyl radical in ethanol.[32] We have recently obtained the rate constant for the reduction of α-tocopheroxyl radical by ubiquinol-10 at 37°C in ethanol as 2.5×10^4 $M^{-1}s^{-1}$.[33] Superoxide-driven reduction of the α-tocopheroxyl radical in the presence of ubiquinone-10 has also been reported.[34]

It has been observed that ubiquinol spares α-tocopherol during lipid peroxidation in solution and liposomal membranes[26,35] and low density lipoprotein.[36–38] The regeneration of α-tocopherol from α-tocopheroxyl radical by ubiquinol in mitochondrial membranes has also been reported.[39,40] α-Tocopheroxyl radical can be reduced by ubiquinol, ascorbate, α-tocopheryl hydroquinone, and dihydrolipoic acid.[41] The relative importance of these reducing compounds in the regeneration of α-tocopherol depends on their concentrations and the active radicals. As shown in Figure 7.5, when phosphatidylcholine is oxidized in liposomal membranes in the presence of ubiquinol-10, α-tocopherol, and ascorbate, the antioxidant was consumed in the order of ascorbate–ubiquinol-10–α-tocopherol when oxidized with a water-soluble radical initiator, while the order was ubiquinol-10–ascorbate_α-tocopherol with a lipid-soluble radical initiator. In both cases, α-tocopherol was spared efficiently. As described above, intermembrane mobility of ubiquinol-10 is restricted and the sparing of α-tocopherol by ubiquinol-10 located in different membranes is not efficient (Figure 7.6).

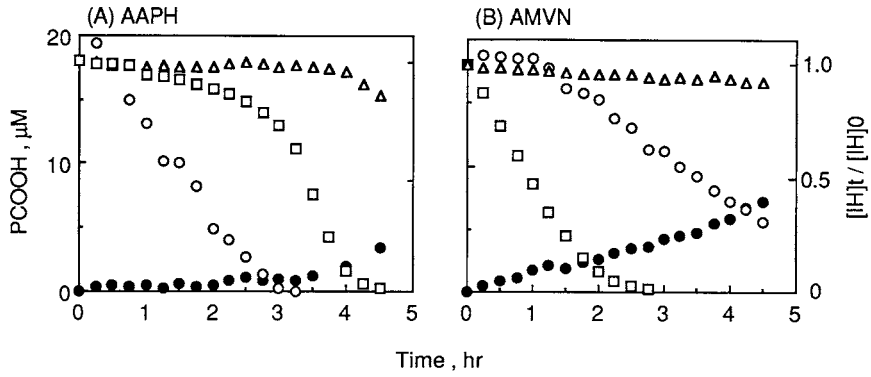

FIGURE 7.5 Consumption of antioxidant (IH) during the oxidation of soybean PC (2.80 mM) liposomes induced by (A) water soluble radical initiator AAPH (1.0 mM), and (B) lipophilic radical initiator AMVN (0.5 mM) at 37°C in air. α-tocopherol (△, 2.5 μM) and ubiquinol (□, 3.0 μM) were incorporated into liposomal membranes, while ascorbic acid (○, 10 μM) was added in the aqueous phase. The consumption of antioxidant and formation (●) of phosphatidylcholine hydroperoxides were followed by HPLC.

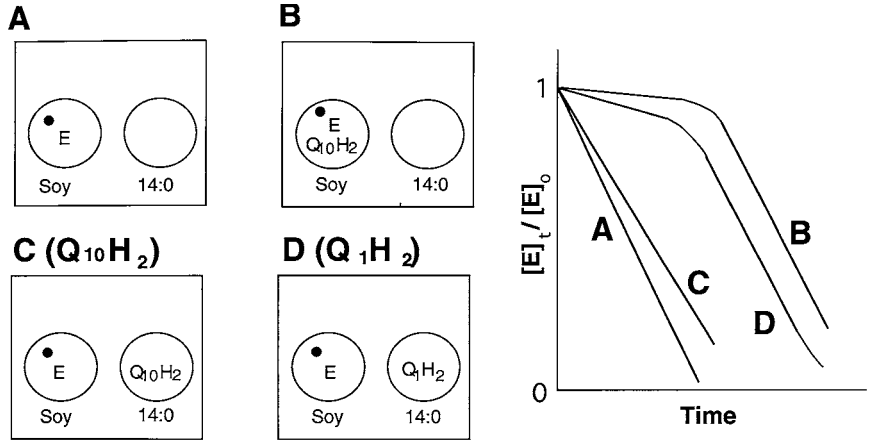

FIGURE 7.6 Effect of location of ubiquinol on the sparing of α-tocopherol. Soybean PC (5.1 mM) multilamellar liposomal membranes containing α-tocopherol (3.0 μM) and AMVN (1.0 mM) were incubated at 37°C in air in the absence and presence of ubiquinol (3.0 μM) and the consumption of α-tocopherol was followed with HPLC. Dimyristoyl PC (5.9 mM) multilamellar vesicles were also incubated together. A: without ubiquinol; B: with ubiquinol-10 incorporated into soybean PC liposomes together with α-tocopherol; C: with ubiquinol-10 incorporated into dimyristoyl PC liposomes; D: with ubiquinol-1 incorporated into dimyristoyl PC liposomes.

7.5 CONCLUSION

There is now ample data showing that coenzyme Q acts as an antioxidant as well as a mobile redox proton carrier in the energy-transducing membranes of mitochondria. A reduced form of coenzyme Q, ubiquinol, is a potent radical scavenger, whereas an oxidized form, ubiquinone, is not. Interestingly, high levels of reduction, 70~100%, have been observed in human tissues, with the exception of brain and lung.[10] It may be possible, and in fact has been observed (see chapters 16 and 17 of this volume by Yamamoto and Kontush), that the degree of reduction is decreased under oxidative stress and pathological conditions, but the redox state should be determined not only by the extent of oxidation, but also by

that of reduction. The enzymatic mechanism for reduction in the mitochondrial inner membrane is well established, but it is not known how or if ubiquinone is reduced in other membranes and lipoproteins. It has been reported that DT-diaphorase maintains the reduced form in the presence of NADH[42] and a novel NADPH-dependent ubiquinone reductase has been found in cytosol.[43] Human blood cells and hepatoma Hep G2 cells have been found to have the capacity to reduce ubiquinone-1, but the rate of reduction of ubiquinone-10 incorporated into LDL was slow.[44] It has also been observed that α-tocopheryl hydroquinone[2,45] and dihydrolipoic acid[46] reduce ubiquinone to ubiquinol.

In spite of numerous studies, the physiological role and significance of ubiquinol as an antioxidant *in vivo* is not yet clear. This is a subject of future study.

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8 Independent and Concerted Antioxidant Functions of Coenzyme Q

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CONTENTS

8.1 INTRODUCTION

Intracellular reductants participate in regulation of oxidative stress through their direct interactions with chain-initiating and/or chain-propagating free radicals and via reduction (enzymatic or non-enzymatic) of molecular products formed from reactive oxygen species or peroxyl radicals, i.e., hydroperoxides. The major protective antioxidant reaction is:

$$AH + ROO\bullet \rightarrow A\bullet + ROOH' \quad (8.1)$$

in which reductants (AH) act as donors of hydrogen for peroxyl radicals (scavengers) to form relatively stable molecular products (hydroperoxides) from reactive chain-propagating radical species at the expense of antioxidant radical (A•) production. An effective biological antioxidant is the one that: (i) effectively scavenges peroxyl radicals, and (ii) whose radicals are not reactive enough to attack important biomolecules. In biomembranes, vitamin E is a good example of an effective antioxidant whose topography in membrane provides not only for effective reaction with polar peroxyl radicals, but is also optimized for important interactions with other reducing antioxidants (such as ascorbate) to completely prevent potential interactions of vitamin E radicals with membrane constituents.[1] As has been demonstrated by Stocker et al.,[2] in the absence of this important "quenching" of vitamin E radicals by reductants in plasma, tocopherols can promote rather than inhibit lipid peroxidation in lipoproteins (see chapter 10 of this volume).

Coenzyme Q (CoQ) is another lipid-soluble compound ubiquitously present in essentially all types of membranes. Since most of CoQ in tissues is reduced, it is capable of donating hydrogen to reactive radicals, and suggests that it may function as a membrane antioxidant along with vitamin E.[3] More than 30 years ago, Mellors and Tappel suggested that there is some kind of interaction between

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these two hydrophobic reductants that may reflect on their antioxidant functions.[4,5] The nature of the interactions between coenzyme Q and vitamin E and their role in antioxidant protection of cell membranes against oxidative stress is the subject of this chapter.

8.2 ANTIOXIDANT FUNCTION OF VITAMIN E AND ITS RECYCLING

The major physiological and biochemical function of vitamin E in membranes and lipoproteins is believed to be the scavenging of reactive oxygen species and free radicals to provide for antioxidant protection.[6] While ubiquitous in different types of biomembranes, vitamin E is, however, a minor component among their lipid constituents. Moreover, antioxidant reactions of vitamin E result in its oxidative degradation, i.e., depletion of vitamin E reserves in the lipid bilayer of membranes. Therefore, relatively low concentrations of vitamin E in membranes cannot fully explain its high effectiveness in antioxidant protection, unless recycling of vitamin E from its free radical intermediates and/or oxidation products occurs.

Recently, antioxidant recycling has been identified as a potentially important mechanism of antioxidant augmentation.[7] In particular, recycling of vitamin E from its phenoxyl radical by vitamin C (ascorbate) was demonstrated both in different model systems and *in vivo*. While in extracellular environments ascorbate-driven recycling of vitamin E may play a very important role, relatively low concentrations of ascorbate in cells suggest that additional pathways may be involved in the process. In particular, the role of thiols [e.g., glutathione (GSH)] in antioxidant recycling has been suggested and subsequently questioned due to low reactivity of thiols toward vitamin E phenoxyl radicals.[8]

A plethora of studies have demonstrated that vitamin E and coenzyme Q are the two major lipid-soluble antioxidants of membranes and lipoproteins. In virtually every location that vitamin E is found, coenzyme Q is also found.[9] There is no doubt that these two compounds both exert antioxidant effects. Their concentrations in membranes are comparable; the reactivities of reduced coenzyme Q (ubiquinol) and vitamin E (tocopherol) toward peroxyl radicals are not significantly different.[10,11,12] Therefore, either of them alone can provide significant antioxidant protection of the membrane lipid bilayer. What are the reasons for having two similar antioxidants in membranes?

The results of our previous work and that conducted by others indicates that when reduced coenzyme Q and vitamin E coexist, coenzyme Q will act as an antioxidant indirectly, by regenerating vitamin E from the vitamin E radical, thus recycling vitamin E for another round of its participation in scavenging reactive radicals, i.e., chain-breaking. Let us consider direct antioxidant reactions of vitamin E and coenzyme Q in greater detail.

8.3 DIRECT ANTIOXIDANT FUNCTION OF COENZYME Q

For many years, studies of CoQ's function in mitochondrial respiration overshadowed persistent reports of CoQ in other membrane fractions in which its function was not readily apparent. It is found in plasma membranes, in all intracellular membranes, and in lipoproteins.[13,14,15] Its concentration is very high in Golgi membranes and in lysosomal membranes (higher, in fact, than in mitochondria), where its function cannot be rationalized in terms of energy-transducing activity.[13] It is also found in lipoproteins, despite the fact that it is manufactured intracellularly and need not be transported between cells.

There is a substantial amount of experimental data showing that coenzyme Q, in addition to its role in electron transport, functions as an antioxidant in its reduced forms in various biological membranes and in low density lipoproteins (LDL) (for reviews see [16, 17]). In 1962, Lea and Kwietney reported that coenzyme Q functioned as an antioxidant.[18] While some experiments suggest that both ubiquinones and ubiquinols might function as antioxidants,[19] other studies have shown that very high concentrations of ubiquinone are required to exhibit significant antioxidant activity,[4,5,20]

and it is the reduced form of the compound that is considered to be the major antioxidant. In pioneering studies, Mellors and Tappel showed that ubiquinol 6 was efficient in inhibiting lipid peroxidation and that electron transport-driven reduction of ubiquinone to ubiquinol resulted in pronounced inhibition of lipid peroxidation in mitochondria.[4,5] Numerous subsequent studies in liposomes,[9,19,20,21,22,23,24,25] mitochondria, microsomes, and submitochondrial particles[26,27,28,29,30,31,32,33,34,35] and cells[36] have established that ubiquinol is capable of inhibiting lipid peroxidation in biological membranes, and that electron transport systems in membranes[30,37] and cytosol,[9] can reduce ubiquinone to ubiquinol. In biological membrane systems in which vitamin E has been removed by mild nondenaturing extraction with pentane,[33] ubiquinol was able to exert antioxidant effects against lipid peroxidation. These results demonstrate that direct antioxidant action of ubiquinol in biological systems is certainly possible.

8.4 COMPARISON OF DIRECT RADICAL SCAVENGING EFFECTS OF VITAMIN E AND COENZYME Q

Thus, both vitamin E and reduced coenzyme Q act as direct chain-breaking antioxidants by donating an H-atom to reduce peroxy- and/or alkoxy-radicals:

$$\mathrm{CoQH} + \mathrm{ROO}^{\bullet} \rightarrow \mathrm{CoQ}^{\bullet} + \mathrm{ROOH} \tag{8.2}$$

where CoQH = ubiquinol, and $\mathrm{CoQ}^{\bullet}$ = ubisemiquinone radical.

However, the chemical reactivity of ubiquinols with peroxyl radicals in organic solvents is slightly lower ($k = 3.4 \times 10^5\ \mathrm{M^{-1}s^{-1}}$) than the reactivity of tocopherols ($k = 33.0 \times 10^5\ \mathrm{M^{-1}s^{-1}}$) ([10, 11, 12], see also chapter 3 in this volume). Since concentrations of free ubiquinols in membranes are roughly equal to or lower than those of vitamin E[38,39] and their concentration in LDL is roughly 10–20% that of vitamin E,[40] it is likely that vitamin E is more efficient as a chain-breaking antioxidant. Indeed, effectiveness of direct peroxyl radical scavenging or antioxidant activity of long-chain ubiquinols (e.g., $\mathrm{CoQ_{10}}$ and $\mathrm{CoQ_9}$) in membranes *in vitro* is lower than that of vitamin E.[31] This again raises the question as to whether direct radical scavenging is in fact an important function for CoQ in antioxidant protection.

8.5 COENZYME Q FACILITATES VITAMIN E RECYCLING

Another possibility for the antioxidant function of coenzyme Q is its interplay with vitamin E resulting in electron transport-driven recycling of vitamin E. The one-electron redox potential for ubiquinol/ubisemiquinone [$E_{7.0}$ ($QH_2/Q^{\bullet}$) = −0.24V] is more negative than that for tocopherol/tocopherol phenoxyl radical [$E_{7.0}$ (T-OH/T-$O^{\bullet}$) = 0.48V][41] suggesting that ubiquinol may reduce the vitamin E phenoxyl radical (formed when vitamin E quenches a peroxyl radical), thus regenerating vitamin E:

$$\mathrm{CoQH} + \mathrm{T\text{-}O}^{\bullet} \rightarrow \mathrm{CoQ}^{\bullet} + \mathrm{T\text{-}OH} \tag{8.3}$$

where T-$O^{\bullet}$ = vitamin E phenoxyl radical and T-OH = vitamin E.

In organic solvents, the interaction of ubiquinols with vitamin E phenoxyl radicals is very efficient: the rate constant for this reaction is about $10^6\ \mathrm{M^{-1}s^{-1}}$,[11] i.e., higher than the rate constant for the reaction of ubiquinol with peroxyl radicals. Thus ubiquinols should preferentially reduce tocopheroxyl radicals rather than peroxyl radicals.

Electron carriers in rat liver microsomes, mitochondria, and submitochondrial particles, as well as in mitochondrial complexes integrated into liposomes, can regenerate tocopherol from its phenoxyl

radical.[31,37,42] In rat liver microsomal membranes, NADPH-dependent electron transport caused transient disappearance of vitamin E phenoxyl radical ESR signals (produced by lipoxygenase/linolenic acid or UV-light) due to reduction of the radical by electron transport.[31] Most importantly, this effect was dramatically enhanced when exogenous CoQ homologues were added to the incubation system to facilitate reduction of the vitamin E phenoxyl radicals. Similarly, succinate-driven reduction of vitamin E radicals in submitochondrial particles was achieved only in the presence of CoQ.[37] Based on the effects of succinate on the levels of CoQ and vitamin E during autooxidation of bovine and rat heart mitochondria, Lass and Sohal concluded that vitamin E acts as the direct radical scavenger, whereas CoQH regenerates vitamin E.[43] These results suggest that redox interactions of coenzyme Q with vitamin E may be important in the antioxidant protection of electron transport membranes. In fact, NADPH-dependent electron transport-supported reduction of CoQ rendered protection of microsomal membranes against azo-initiator-induced lipid peroxidation in a synergistic fashion with vitamin E.[44] Hence, in addition to CoQ's direct antioxidant effects, its interaction with vitamin E—realized through its reduction of vitamin E phenoxyl radicals—leads to vitamin E recycling and more effective antioxidant protection in biological systems.

The importance of the mechanism is that it links the reduction of coenzyme Q and, hence recycling of vitamin E, to membrane electron transport systems and other enzymes capable of reducing CoQ to CoQH (e.g., lipoamide dehydrogenase).[45] In other words, this interaction couples vitamin E recycling to the major metabolic pathways—enzymes of electron transport in mitochondria, endoplasmic reticulum, and plasma membranes. As a result, the antioxidant effectiveness of vitamin E is enhanced manyfold. This, however, only explains why vitamin E needs coenzyme Q for its successful functioning as a physiological membrane antioxidant. Is there any need for vitamin E in coenzyme Q's antioxidant role?

8.6 COENZYME Q SEMIQUINONE RADICAL REDUCES VITAMIN E PHENOXYL RADICAL

Electron transport in membranes is always accompanied by generation and release of superoxide, whose level may become very high under some conditions (e.g., during the oxidative burst of phagocytic cells, or in the presence of redox-cycling drugs).[14,46] Overproduction of superoxide creates prooxidant conditions, resulting in depletion of antioxidants and development of oxidative stress.

Superoxide readily reduces coenzyme Q to form ubisemiquinone and ubiquinol, directly or via disproportionation of ubisemiquinone radicals, (Equations 8.4, and 8.5):[47,48]

$$CoQ + O_2^{\cdot-} \rightarrow CoQH^{\cdot} + O_2 \tag{8.4}$$

$$2CoQ^{\cdot} + H^{+} \rightarrow CoQH + CoQ \tag{8.5}$$

where CoQ = ubiquinone, $CoQ^{\cdot}$ = ubisemiquinone, and CoQH = ubiquinol.

The reaction (8.4) is reversible—ubisemiquinone can undergo autooxidation yielding superoxide radicals.[49,50] This suggests that the ubiquinone/ubisemiquinone redox-couple may exert either antioxidant or prooxidant effects depending on the steady-state concentrations of oxidized and reduced coenzyme Q, oxygen, and superoxide. It should also be noted that ubisemiquinone is formed every time reduced coenzyme Q acts as a direct chain-breaking antioxidant (Figure 8.1). Clearly, production of superoxide by an antioxidant molecule is not consistent with its protective antioxidant role. If, however, $CoQ^{\cdot}$ and $O_2^{\cdot-}$ are both consumed by reaction(s) with another antioxidant, then the propensity of CoQ to be reduced to CoQ• may serve a very important antioxidant role.

That is exactly what vitamin E and its one-electron oxidation product, vitamin E phenoxyl radical, can do under conditions of oxidative stress. If oxidative stress is induced by superoxide, two redox-events

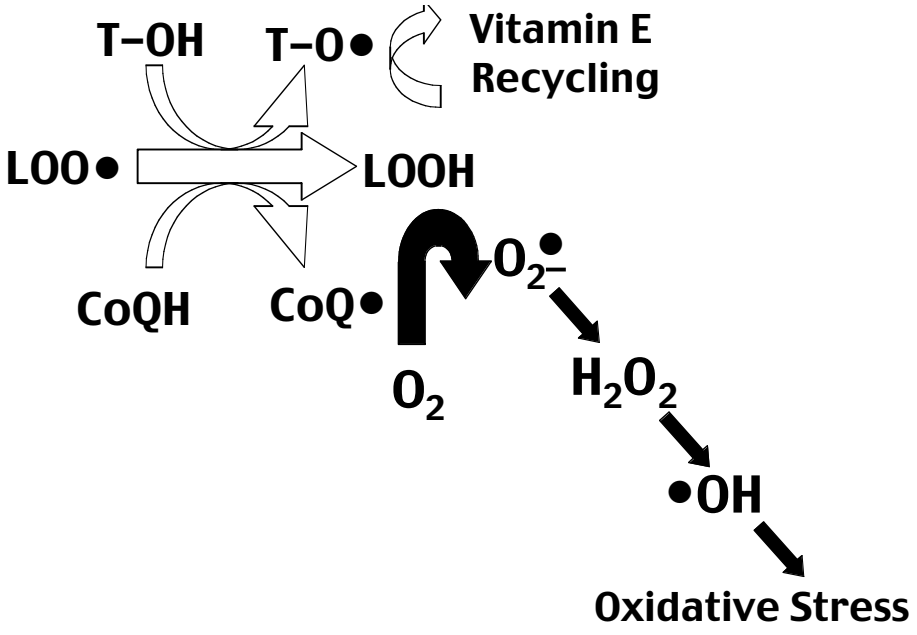

FIGURE 8.1 Scheme illustrating radical scavenging reactions of tocopherol and ubiquinol. Note the formation of ubisemiquinone that can donate an electron to molecular oxygen to produce superoxide anion-radical, hence triggering oxidative stress. CoQ•—ubisemiquinone, CoQH—ubiquinol, T-OH—tocopherol, T-O•—tocopherol phenoxyl radical.

will occur. One is superoxide-driven reduction of CoQ to CoQ•. The other is oxidation of vitamin E to its phenoxyl radical:

$$\text{E-OH} + \text{O}_2^{\bullet -} \rightarrow \text{E-O}^{\bullet} + \text{O}_2 + \text{H}^+ \tag{8.6}$$

Therefore, vitamin E and its one-electron oxidation intermediate formed in the course of radical scavenging activity of vitamin E, its phenoxyl radical, can act as electron sinks. This allows for CoQ• to donate its electron for productive recycling of vitamin E rather than for generating superoxide anion and other reactive oxygen species, i.e., trigger prooxidant cascades (Figure 8.2).

Our initial experiments utilized simple and well-defined superoxide generating model systems—K_2O ·/crown ether in an aprotic medium (DMSO) and xanthine/xanthine oxidase in aqueous systems.[51] We demonstrated that CoQ_{10} protected vitamin E against oxidation by superoxide in a concentration-dependent manner. Vitamin E was oxidized by superoxide to form ESR-detectable radicals of tocopherol semiquinone radicals. In the presence of CoQ_{10}, neither these radicals, nor vitamin E phenoxyl radicals (generated by UV-light, or PbO_2) could be detected in ESR spectra. Instead, ESR signals of CoQ_{10} semiquinone radicals were observed. Vitamin E caused a concentration-dependent decrease of CoQ_{10} semiquinone radical steady-state concentration. These model experiments in aprotic medium demonstrate that one electron reduction of CoQ_{10} by superoxide ion resulting in the formation of CoQ_{10} semiquinone radicals caused redox-cycling of vitamin E from its phenoxyl radical, thus, preventing loss of vitamin E. This suggests that CoQ (in its oxidized form) is able to prevent superoxide-driven consumption of vitamin E, at least in aprotic medium. We further found this mechanism can effectively operate in aqueous systems as well. Water-soluble CoQ_0 protected a water-soluble homologue of vitamin E, Trolox, against superoxide-induced oxidation (produced by xanthine oxidase/xanthine system). CoQ_0 semiquinone radicals detectable by ESR in the presence of xanthine/xanthine oxidase were no longer present in ESR spectra upon addition

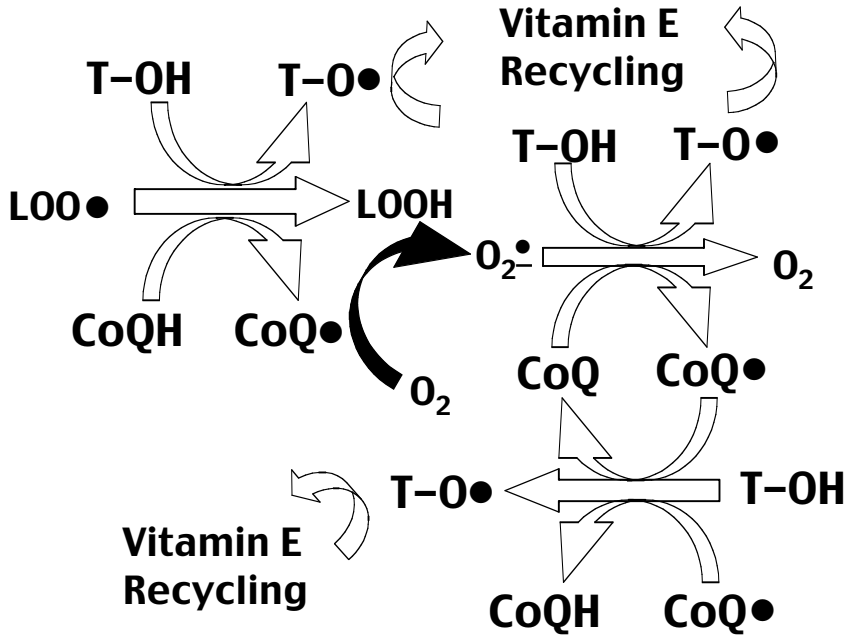

FIGURE 8.2 Scheme illustrating radical scavenging reactions of tocopherol and ubiquinol. Note that reduction of ubiquinone to semiubiquinone utilizes superoxide. Ubisemiquinone formed reduces tocopherol phenoxyl radical i.e., recycles vitamin E. Thus superoxide is productively consumed to recycle vitamin E in CoQ-dependent reactions instead of its potential ability to induce oxidative stress. CoQ—ubiquinone, CoQ•—ubisemiquinone, CoQH—ubiquinol, T-OH—tocopherol, T-O•—tocopherol phenoxyl radical.

of Trolox.[52] Combined, these results indicate that not only CoQH, but also CoQ_{10} semiquinone radicals can reduce vitamin E phenoxyl radical in a one-electron reaction. This suggests that CoQ_{10} may have another important physiological function, i.e., protection of vitamin E against superoxide-driven oxidation.

In our subsequent studies, we tested whether this important mechanism of CoQ_{10} semiquinone radicals may function in membrane systems. For this, we used purified human recombinant NADPH-cytochrome P-450 reductase. We demonstrated that CoQ mediates recycling of vitamin E in a superoxide-driven reaction. We further found that NADPH-cytochrome P-450 reductase reduced phenoxyl radicals of vitamin E and its homologues (e.g., radicals of 2,2,5,7,8-pentamethyl-6-hydroxychromane) in NADPH-dependent reaction both directly and via coenzyme Q/superoxide-driven mechanisms. NADPH-induced (superoxide-driven) recycling of vitamin E by NADPH-cytochrome P-450 oxidoreductase was dependent on the presence of CoQ and was completely inhibited by superoxide dismutase (SOD).[52]

Another membrane electron transport enzyme system, plasma membrane coenzyme Q reductase, can operate in a very similar way. In our joint work with Drs. Navas, Villalba, and Arroyo (University of Cordoba, Spain) we studied CoQ/superoxide/Trolox interactions using purified plasma membrane coenzyme Q reductase.[53] We found that this membrane enzymatic system catalyzed NADH/coenzyme Q_o-dependent reduction of phenoxyl radicals generated by lipoxygenase/linoleic acid from Trolox, a water-soluble homologue of vitamin E. Characteristic ESR spectra of Trolox phenoxyl radicals were not observed when NADH-dependent electron transport was initiated through plasma membrane coenzyme Q reductase in the presence of CoQ. Typical spectra of coenzyme Q_o semiquinone radicals were detected instead. Trolox radical signals reappeared in the spectra after complete consumption of NADH. The reduction of phenoxyl radicals occurred through their interactions with reduced coenzyme Q_o (or its semiquinone radical). Both superoxide-driven

reduction and direct enzyme-mediated reduction of coenzyme Q_o was involved in the recycling of Trolox from its phenoxyl radicals as evidenced by a significant (more than 50%) inhibitory effect of Cu, Zn-SOD. Combined, these results demonstrate that vitamin E phenoxyl radicals are important for effective antioxidant functioning of CoQ because they act as an electron sink through which CoQ semiquinone radicals and superoxide are eliminated to prevent oxidative damage associated with superoxide-induced Fenton chemistry. Interestingly, a recent study demonstrated that vitamin E and selenium deficiency induces expression of the CoQ-dependent plasma membrane reductase system i.e., compensates by enhanced effectiveness of CoQ-dependent antioxidant function.[54]

Finally, we studied whether superoxide-dependent protective effects of CoQ may be realized in cells. We performed measurements of site-specific lipid peroxidation in human leukemia HL-60 cells. We metabolically labeled endogenous phospholipids in cells using an oxidation-sensitive fluorescent fatty acid, *cis*-parinaric acid (PnA), exposed the cells to oxidative stress, and tested for antioxidant protection by CoQ. The cells were supplemented with vitamin E (20 nmol/10^6 cells) and incubated with a lipid-soluble azo-initiator of peroxyl radicals, 2,2'-azobis-2,4-dimethylvaleronitrile, AMVN (Table 8.1). HPLC-fluorescence assay of PnA-labeled phospholipids showed that a pronounced oxidation was induced in four major classes of phospholipids: PC, PE, PS, and PI after exposure to AMVN. When the cells were incubated in the presence of xanthine oxidase/xanthine, even greater oxidation of phospholipids occurred. Expectedly, combination of AMVN and xanthine oxidase/xanthine produced an additive dramatic depletion of PnA-labeled phospholipids. Notably, in the presence of oxidized CoQ a significant protection against (AMVN plus xanthine oxidase/xanthine)-induced oxidation was found. Oxidized CoQ did not cause any significant protection against AMVN-induced oxidation (data not shown). These results strongly suggest that superoxide produced by xanthine oxidase/xanthine caused reduction of CoQ to CoQ• and CoQH, which were able to protect phospholipids against oxidation, likely through cooperative interactions of CoQ and vitamin E.

Moreover, results of a recent study by Lass et al.[55] indicate that long-term administration of CoQ_{10} or α-tocopherol can result in an elevation of their concentrations in the tissues of the mouse. More importantly, CoQ_{10} intake has a sparing effect on α-tocopherol in mitochondria *in vivo*.

The oxidation of plasma lipoproteins is a hallmark of atherosclerosis and may play a potential role in the pathogenesis of vascular remodeling. As mentioned earlier, the role of vitamin E alone

TABLE 8.1
Effect of Superoxide-Driven Reduction of Coenzyme Q10 on Lipid Peroxidation Induced by AMVN in HL-60 Cells Supplemented With α-Tocopherol

	Oxidation of *cis*-PnA (ng/μg) Total Lipid (Pi/hr)			
Treatment	**PI**	**PE**	**PS**	**PC**
AMVN	60.0 ± 41.4	290.4 ± 10.8	34.8 ± 14.4	652.8 ± 207.6
X-Xo	220.8 ± 33.1	698.4 ± 105.6	55.8 ± 10.9	2466.0 ± 309.5
AMVN+X-Xo	325.2 ± 49.8	930.0 ± 163.2	79.2 ± 6.0	3373.2 ± 504.6
AMVN+X-Xo+Q_{10}	284.4 ± 3.0	742.2 ± 75.6	69.0 ± 2.0	2400.0 ± 30.0

HL-60 cells (0.5×10^6) were grown in RPMI medium 1640 supplemented with 10% fetal bovine serum and α-tocopherol (20 nmol/10^6 cells) for 24h at 37°C in CO_2 atmosphere. Cells were harvested by centrifugation, washed, resuspended in buffer and *cis*-parinaric acid was metabolically incorporated into HL-60 cells.

cis-PnA; *cis*-parinaric acid, PC; phosphatidylcholine, PE; phosphatidylethanolamine, PS; phosphatidylserine, PI; phosphatidylinositol, AMVN; 2,2'-azobis-2,4-dimethylvaleronitrile, X-Xo; xanthine-xanthine oxidase.

in protection of lipoproteins from oxidation is controversial since vitamin E can act as an apparent oxidant in the absence of adequate recycling mechanisms.[2] Reduced CoQ (CoQH) represents an important factor in lipoprotein particles that serves to recycle vitamin E via the mechanisms described above. Surprisingly little CoQ, however, could be recovered from LDL present in plasma and even less in its reduced form (CoQH).[56,57] This suggests that recycling of vitamin E by CoQH may be limited in LDL unless CoQ recycling mechanisms are involved. Our proposed mechanisms for superoxide-driven vitamin E radical reduction by CoQ• offers a pathway that may be an essential component for the vitamin E recycling. This mechanism may explain how minimal amounts of CoQ in either reduced or oxidized form can mediate catalytic vitamin E recycling by superoxide. It needs to be pointed out that superoxide formation during the respiratory burst following inflammatory cell activation within the vessel wall may be the primary stimulus for LDL oxidation *in vivo*. Therefore, under these circumstances one might expect significant dynamic shifts in the steady-state concentrations of reduced and semireduced CoQ and, thus, CoQ-mediated augmentation of the protective action of vitamin E towards LDL oxidation in the presence of superoxide.

Most of what we know regarding vitamin E and CoQ interactions has been elucidated using uncomplicated cell-free model systems or grossly simplified cell-derived fractions (i.e., membranes) as described above. Therefore, the functional repercussions of these potential interactions in terms of cell function and their role *in vivo* are just beginning to be explored. One would anticipate that CoQ/vitamin E interactions could profoundly influence mitochondrial function since this organelle represents a major source of electron transport. Cell death, both necrotic and apoptotic, is accompanied by an uncoupling of electron transport and the formation of reactive oxygen species, most notably superoxide. Much recent attention has focused on the role of mitochondrial superoxide production,[58] changes in mitochondrial permeability transition,[59] and cytochrome *c* release[60] as regulators of apoptosis. It is intriguing to speculate that vitamin E/CoQ interaction within the mitochondria could be an arbiter of cell fate and determine whether the cell follows a necrotic or apoptotic pathway to its ultimate demise following exposure to noxious stimuli.[61,62]

An interesting set of observations made by Dr. Navas and colleagues involves the association between plasma membrane NADH/CoQ reductase and cell survival following growth factor withdrawal. First, the addition of CoQ, as well as traditional antioxidants such as vitamins E and C to cells attenuates the extent of apoptosis following serum withdrawal.[63] Secondly, HL-60 cells made deficient in functional mitochondria (ρ° HL-60) by prolonged culture in the presence of ethidium bromide possessed enhanced CoQ content and expression of CoQ reductase activity ([64], see also chapter 5 in this volume). Interestingly, these ρ° HL-60 cells were resistant to apoptosis following growth factor withdrawal. This protection against cell death appeared to be related to the decreased ability of these cells to activate neutral sphingomyelinase and accumulate ceramide.[65] Since ceramide is an important intracellular mediator of intracellular communication that serves to inhibit electron transport in mitochondria and increase H_2O_2 production by this organelle,[66] vitamin E/CoQ interactions at the level of the plasma membrane may regulate important signal transduction pathways. Of course it can be argued that this effect is not specific for CoQ since these changes could have arisen simply as an artifact of selection. Therefore, definitive demonstration of the importance of this relationship awaits the application of technology that will allow the specific manipulation of this system in order to test its effects. In this regard, identification and cloning the genes responsible for CoQ synthesis and creation of mutant yeast strains deficient in these pathways will most certainly yield fruitful approaches ([67, 68], see also Chapter 7 in this volume).

In conclusion, vitamin E and CoQ form an essential redox-couple whose concerted action provides for an effective antioxidant protection of membranes and lipoproteins. Vitamin E antioxidant effectiveness is enhanced manyfold by CoQ, which links vitamin E recycling process to major metabolic electron transport mechanisms. Reciprocally, vitamin E and its phenoxyl radical are absolutely crucial for coenzyme's antioxidant function because they prevent accumulation of ubisemiquinone radicals leading to dangerous (in the absence of vitamin E) prooxidant effects of coenzyme Q.

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9 Mechanisms of Antioxidant Action of Ubiquinol-10 for Low-Density Lipoprotein

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CONTENTS

Increasing evidence implicates oxidative modification of low-density lipoprotein (LDL) as an important event contributing to atherogenesis. The "oxidative theory" of atherosclerosis implies that antioxidants are anti-atherogenic. LDL contains various antioxidants including α-tocopherol (α-TOH) and ubiquinol-10 ($CoQ_{10}H_2$). As the most abundant lipid-soluble antioxidant present in plasma lipoproteins, α-tocopherol (α-TOH) has received the most attention with respect to studies investigating the molecular mechanisms of LDL lipid peroxidation and antioxidation. It has been established that α-TOH in LDL does not act in its conventional role as a chain-breaking antioxidant. Instead, α-TOH can exhibit antioxidant or prooxidant activity for lipoprotein lipids depending on the degree of radical flux and reactivity of the oxidant to which LDL is exposed. To explain the complex molecular action of α-TOH during lipoprotein lipid peroxidation and antioxidation, the model of tocopherol-mediated peroxidation (TMP)

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has been formulated. TMP proposes that the prooxidant activity of α-TOH is prevented by coantioxidants, compounds capable of reducing the α-tocopheroxyl radical and exporting the radical from the lipoprotein particle. $CoQ_{10}H_2$ represents an effective lipophilic coantioxidant. Consistent with this, $CoQ_{10}H_2$ reduces the α-tocopheroxyl radical and is the first antioxidant consumed in LDL exposed to various oxidizing conditions. Furthermore, dietary enrichment of LDL with $CoQ_{10}H_2$ protects lipoprotein lipids against *ex vivo* oxidation. This chapter outlines the salient features of TMP, discusses how low levels $CoQ_{10}H_2$ inhibit LDL lipid peroxidation, and how supplementation with $CoQ_{10}H_2$ may be required for α-TOH to more efficiently attenuate lipoprotein lipid oxidation *in vivo*, and perhaps atherogenesis.

9.1 INTRODUCTION

Atherosclerosis, a disease of arteries, is a major cause of morbidity and mortality in the Western world. Rupture of an advanced atherosclerotic lesion results in occlusion of the affected artery. Formation of the atherosclerotic lesion is thought to result from a complex and excessive chronic inflammatory-fibroproliferative response of the vascular wall to endothelial injury/dysfunction,[1] excessive retention of low-density lipoprotein (LDL) in the arterial wall,[2] and oxidative modification of LDL.[3]

The "oxidation theory" proposes that accumulation of oxidized LDL in the subendothelial space of arteries represents an important causative event for atherogenesis and that antioxidants are potential antiatherogenic compounds.[3] In support of this, oxidized lipoproteins are detected in atherosclerosic lesions[4–7] and *in vitro* oxidized LDL exhibits potential proatherogenic activities that may participate in both early and late events of atherogenesis.[3,8] Thus, oxidatively modified, but not native, LDL is taken up by macrophages in an uncontrolled manner via scavenger receptors to form lipid-laden or "foam" cells,[9] the hallmark of early atherosclerotic lesions. There is evidence that scavenger receptors are important for the development of atherosclerosis in certain experimental animal models of the disease.[10] However, it is not clear whether, and if so, how, this relates to foam cell formation and/or oxidized LDL. *In vitro* oxidized LDL can also promote endothelial dysfunction, the attraction to and retention of blood monocytes within the artery wall, cell proliferation and cytotoxicity, thrombogenic reactions, and expression of matrix degrading enzymes.[3,8] However, the true physiological relevance of such *in vitro* oxidized LDL remains to be established. Further support for the oxidation hypothesis is that certain (though not all) antioxidants inhibit the extent of atherosclerosis in experimental animals. It has been proposed that antioxidants act primarily by inhibiting lipoprotein oxidation.[3] However, recent studies indicate that antioxidants may modulate atherosclerosis via actions in addition to, or independent of, their effects on LDL oxidation.[11,12]

Due to the proatherogenic potential of oxidized LDL, many studies have focused on understanding the molecular mechanisms of LDL oxidation and its prevention by antioxidants. Human LDL contains various endogenous antioxidants of which α-TOH and ubiquinol-10 ($CoQ_{10}H_2$; the reduced and antioxidant form of coenzyme Q_{10}) appear to be the most important with respect to modulation of LDL lipid peroxidation.[13–20] α-TOH, biologically and chemically the most active form of vitamin E,[21] represents the major lipid-soluble antioxidant present in extracts prepared from human lipoproteins. LDL contains, on average, 6 to 12 molecules of α-TOH per lipoprotein particle[15,22] and $\approx$0.5 to 1 molecule of $CoQ_{10}H_2$ per lipoprotein particle* (Table 9.1). Accordingly, α-TOH has received most attention with respect to research into inhibition of LDL lipid peroxidation and as a potential antiatherogenic supplement. However, results of vitamin E intervention studies on the extent of atherosclerosis in experimental animals, and of controlled prospective trials on the incidence of cardiovascular disease in humans, have, overall, been inconclusive.[11,23,24] Furthermore, an increasing number of *in vitro* studies[15,17–19,25–31] have demonstrated that α-TOH can exert

* A commonly cited review article [22] quotes ≈ 0.1 $CoQ_{10}H_2$ per LDL. However, these authors, despite measuring ubiquinone, did not take special care to preserve $CoQ_{10}H_2$ during LDL isolation and extraction. Due to its labile nature, it is likely that a majority of $CoQ_{10}H_2$ autoxidized to CoQ_{10} and hence was not accurately assessed.

TABLE 9.1
Composition of Human LDL[a]

Component	Weight (%)	mol/LDL
Protein		
Apoprotein B-100	22.0 ± 1.9	1
Lipids		
Phospholipids	22.3 ± 3.9	700
Phosphatidylcholine		450
Bisallylic hydrogens[a]		375
Cholesterol	9.6 ± 0.7	600
Cholesterylesters	42.2 ± 3.8	1600
Cholesteryllinoleate		880
Cholesterylarachidonate		95
Bisallylic hydrogens[a]		1165
Triglycerides	5.9 ± 2.7	180
Bisallylic hydrogens[a]		50
Total bisallylic hydrogens[a]		1590
Antioxidants		
α-Tocopherol		6–12
γ-Tocopherol		0.5
Ubiquinol-10		0.5–0.8[b]
Lycopene		0.2–0.7
β-Carotene		0.1–0.4

[a] The values given are derived from.[14,15,22,112,113]

[b] Bisallylic hydrogens refer to the polyunsaturated fatty acids in LDL, i.e., the most readily oxidized lipid moieties of the lipoprotein. Linoleic and arachidonic acid contain one and three pairs of bisallylic hydrogens, respectively.

[c] These values have been determined in [16].

prooxidant activity for lipoprotein lipids, in direct conflict with the view that the vitamin acts in its conventional mode as a chain-breaking antioxidant.

The model of tocopherol-mediated peroxidation (TMP) has been developed to explain the actions of α-TOH during lipoprotein lipid peroxidation.[15] TMP proposes that α-TOH facilitates the transfer of aqueous radicals into LDL and its one-electron oxidation product, α-tocopheroxyl radical (α-TO$^{\bullet}$), acts as a peroxidation chain-transfer agent *causing* formation of lipid hydroperoxides in lipoproteins. Compounds capable of reducing the α-TO• radical and exporting the radical from the lipoprotein particle, termed coantioxidants,[32] prevent plasma lipoprotein lipid peroxidation. In this chapter we outline evidence indicating that $CoQ_{10}H_2$ represents an effective lipophilic coantioxidant for plasma lipoproteins, and discuss whether TMP and coantioxidation are relevant for aortic lipid peroxidation occurring during atherogenesis.

9.2 MOLECULAR ACTIONS OF VITAMIN E DURING LIPOPROTEIN LIPID PEROXIDATION

LDL is a spherical particle of 20 to 22 nm diameter consisting of a molecule of apoprotein B-100 (apo B) embedded in a monolayer "surface" of polar phospholipids and cholesterol that surrounds a "core" of neutral cholesteryl esters and triglycerides (Table 9.1). The oxidizability of LDL lipids

is primarily dependent on their content of bisallylic hydrogen atoms. Cholesteryl esters contain ≈ threefold more bisallylic hydrogens than phospholipids and as such are the major lipid substrates for peroxidation in LDL (Table 9.1). LDL lipid peroxidation is generally held to proceed, and to a certain extent cause, the oxidation of apo B[33] and oxidized lipid possess various proatherogenic activities.[8,34] Therefore, understanding the molecular events involved in LDL lipid peroxidation and how antioxidants prevent this process may provide important information in designing antioxidant strategies to attenuate oxidative modification of lipoproteins *in vivo* and perhaps atherosclerosis. Importantly, however, certain oxidants (e.g., HOCl[35] and peroxynitrite[31]) directly oxidize apo B independent of lipid peroxidation. Therefore, different antioxidant strategies may be required to adequately protect both lipoprotein lipid and protein moieties from oxidative modification *in vivo*.

Before discussing the molecular mechanisms of lipoprotein lipid peroxidation (i.e., complex heterogeneous lipid emulsions) and the role of α-TOH and $CoQ_{10}H_2$ in this process, we will first briefly review the features of lipid oxidation and antioxidation occurring in homogeneous systems and liposomes.

9.2.1 Radical Scavenging Activity of α-TOH and $CoQ_{10}H_2$

Studies in homogeneous solutions and liposomes have established that α-TOH[36] and $CoQ_{10}H_2$[37–39] are effective lipophilic chain-breaking antioxidants and as such effectively suppress lipid peroxidation. Thus, α-TOH and $CoQ_{10}H_2$ rapidly react with the chain-carrying lipid peroxyl radical ($LOO^{\bullet}$) (Reactions 9.1 and 9.2). Alternatively, α-TOH and $CoQ_{10}H_2$ can react directly with the peroxidation initiating peroxyl radical ($ROO^{\bullet}$) (Reactions 9.3 and 9.4). Radical scavenging by α-TOH and $CoQ_{10}H_2$ results in the formation of the relatively unreactive α-tocopheroxyl radical (α-$TO^{\bullet}$) and the protonated ubisemiquinone radical ($CoQ_{10}H^{\bullet}$; a para hydroxy substituted phenoxyl radical), respectively.

$$\alpha\text{-TOH} + LOO^{\bullet} \rightarrow \alpha\text{-}TO^{\bullet} + LOOH \tag{9.1}$$

$$CoQ_{10}H_2 + LOO^{\bullet} \rightarrow CoQ_{10}H^{\bullet} + LOOH \tag{9.2}$$

$$\alpha\text{-TOH} + ROO^{\bullet} \rightarrow \alpha\text{-}TO^{\bullet} + ROOH \tag{9.3}$$

$$CoQ_{10}H_2 + ROO^{\bullet} \rightarrow CoQ_{10}H^{\bullet} + ROOH \tag{9.4}$$

The chain-breaking action of α-TOH and $CoQ_{10}H_2$ results in a well-defined "lag period" during which less than one mole of lipid hydroperoxide (LOOH) is formed per mole of α-TOH or $CoQ_{10}H_2$ consumed (i.e., radical chain length $\nu < 1.0$). The length of the lag phase is increased when α-TOH and $CoQ_{10}H_2$ are added to liposomes in combination[38,39] and $CoQ_{10}H_2$ is consumed before α-TOH in this system.[38,39] As the rate constants for the reaction of peroxyl radicals and α-TOH or $CoQ_{10}H_2$ are comparable, a "sparing" effect of $CoQ_{10}H_2$ for α-TOH suggests a reduction of α-$TO^{\bullet}$ by $CoQ_{10}H_2$. Studies in organic solution,[40] liposomes,[41] or autoxidizing mitochondrial membranes[42] have provided more direct support for the reduction of α-$TO^{\bullet}$ by $CoQ_{10}H_2$.

Various studies have reported that in liposomes, α-TOH prevents lipid peroxidation more efficiently than $CoQ_{10}H_2$.[38,43,44] The lower antioxidant efficacy of $CoQ_{10}H_2$ is likely due to its greater propensity to autoxidize (via $CoQ_{10}H^{\bullet}$ and the ubisemiquinone radical, $CoQ_{10}^{\bullet -}$), a process that requires protons (H^+)[45] (Reactions 9.5 and 9.6).

$$CoQ_{10}H^{\bullet} \leftrightarrow CoQ_{10}^{\bullet -} + H^+ \tag{9.5}$$

$$CoQ_{10}^{\bullet -} + H^+ + O_2 \rightarrow CoQ_{10} + O_2^{\bullet -} + H^+ \tag{9.6}$$

Such autoxidation likely explains why the stoichiometric number for $CoQ_{10}H_2$ in liposomes is ≈ 1.[38] The lower antioxidant efficiency of $CoQ_{10}H_2$ may also derive from a competing prooxidant activity of $CoQ_{10}H^{\bullet}$.[44,46] Thus, $CoQ_{10}H^{\bullet}$ may autoxidize in an aprotic, lipophilic environment to give rise to the hydroperoxyl radical ($^{\bullet}OOH$) (Reactions 9.6), which itself can oxidize lipids.[47] Furthermore, a study in organic solvents has suggested that $CoQ_{10}H^{\bullet}$ can promote homolysis of H_2O_2 or LOOH to form more highly reactive $^{\bullet}OH$ and alkoxyl radicals, respectively.[44]

Although studies in homogeneous solution and liposomes can provide valuable information, caution is required in the extrapolation of such results to biological membranes or lipoproteins. First, the ratio of coenzyme Q to lipid employed in the liposomal systems is extremely high and nonphysiological. Second, the precise location and orientation of coenzyme Q in liposomes is unknown and may be different from that in biomembranes. In biomembranes, coenzyme Q can interact with proteins and there is evidence that at least in mitochondria, membrane proteins can bind and stabilize the ubisemiquinone radical.[48]

9.2.2 The Role of α-TOH and $CoQ_{10}H_2$ in LDL Lipid Peroxidation is Dependent on the *In Vitro* Oxidizing Conditions Employed

Similar to the situation in homogeneous solutions, a clearly defined initial period of low rates of lipid peroxidation is observed when isolated LDL is oxidized by exposure to high and nonphysiological concentrations of Cu^{2+}.[49] During this initial period $CoQ_{10}H_2$, α-TOH, and other compounds referred to as "antioxidants" (e.g., carotenoids) are consumed rapidly. Following complete consumption of these antioxidants, lipid peroxidation proceeds at high rates.[49] These results *suggested* that α-TOH represents an effective chain-breaking antioxidant for LDL's lipids *in vitro*. Consistent with this, supplementation of the lipoprotein with α-TOH increases the length of the "lag phase," when LDL is exposed to these strongly oxidizing conditions.[13,50,51]

However, many studies have documented a lack of significant correlation between α-TOH content and duration of lag time when native LDL is exposed to high Cu^{2+} concentrations.[19,20,52,53] Furthermore, when $CoQ_{10}H_2$-free lipoproteins and ascorbate- and $CoQ_{10}H_2$-free plasma are exposed to more mild oxidizing conditions, α-TOH promotes, and is even required for efficient initiation of lipid peroxidation.[17,54] Under such mild oxidizing conditions, lipoprotein lipid peroxidation in $CoQ_{10}H_2$-free lipoproteins (i) proceeds via a radical chain reaction of length > 1 in the presence of α-TOH; (ii) is accelerated by enriching the LDL with α-TOH, (iii) is markedly suppressed in LDL deficient in α-TOH; and (iv) is faster in the presence of α-TOH than immediately after its complete consumption.[14,15,17,18,25,28,31,55] These findings are not consistent with the conventional view that vitamin E acts as a chain-breaking antioxidant for LDL lipids.

9.2.3 Tocopherol-Mediated Peroxidation

A kinetic analysis of LDL lipid peroxidation initiated by $ROO^{\bullet}$ resulted in the formulation of TMP[15] as a general model to explain the molecular events involved in lipid peroxidation and antioxidation in isolated, α-TOH-containing LDL exposed to radical oxidants (Figure 9.1). The TMP model of lipid peroxidation encompasses the physical constraints and consequences of the radical reactions taking place in emulsions of peroxidizing lipoproteins. The model predicts that α-TOH, in the absence of $CoQ_{10}H_2$ and other low-molecular weight antioxidants (see below) can promote LDL lipid peroxidation. Principally, this is due to both the phase-transfer activity of α-TOH (Reaction 9.1, Figure 9.1) and the chain-transfer activity of α-$TO^{\bullet}$ (Reaction 9.2, Figure 9.1).[15,25,56,57] TMP and the molecular action of vitamin E in oxidizing lipoproteins have been reviewed recently.[23,24,58–61,62]

The *in vitro* oxidizing conditions employed determine whether α-TOH acts as an antioxidant or a prooxidant for lipids in $CoQ_{10}H_2$-free, isolated LDL. Under conditions of high radical flux, radical-radical termination reactions between α-$TO^{\bullet}$ and the oxidation-initiating radical predominate (Reaction 9.5, Figure 9.1), such that α-TOH exhibits an overall antioxidant activity. This readily

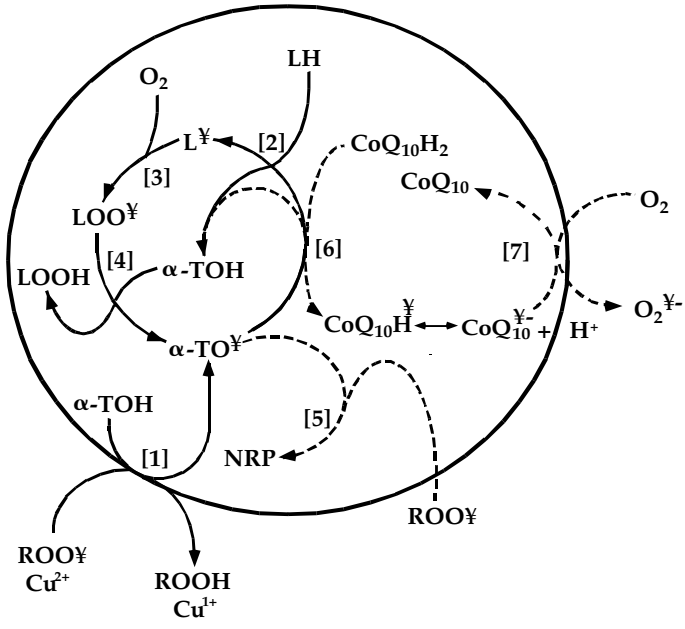

FIGURE 9.1 Model of TMP for LDL lipid oxidation and antioxidation by $CoQ_{10}H_2$. A solution of radical oxidizing lipoprotein is an aqueous emulsion of lipid particles where the radical in one oxidizing particle, present predominantly as α-TO$^{\bullet}$, is segregated from α-TO$^{\bullet}$ in other oxidizing particles, and oxidation of the lipids proceeds via TMP.[15] TMP (solid lines) is initiated by Reaction 9.1, reflecting the phase-transfer activity of α-TOH. Lipid peroxidation initiation (Reaction 9.2), followed by the propagation Reactions 9.3 and 9.4, reflect the chain-transfer activity of α-TO$^{\bullet}$. This is a feature relevant for LDL exposed to mild radical fluxes. Inhibition of TMP (anti-TMP, broken lines) can be achieved by reaction of a second aqueous radical oxidant with α-TO$^{\bullet}$ (Reaction 9.5), resulting in both formation of nonradical product(s) (NRP) and consumption of α-TOH. This is a feature particularly relevant to high radical flux conditions, where α-TOH appears to act as a conventional antioxidant. Alternatively, anti-TMP is achieved by LDL-associated $CoQ_{10}H_2$, (or other coantioxidants), which reduces α-TO$^{\bullet}$ (Reaction 9.6) resulting in the formation of $CoQ_{10}H^{\bullet}$, which may undergo one of two reactions. First, $CoQ_{10}^{\bullet-}/CoQ_{10}H^{\bullet}$ may scavenge α-TO$^{\bullet}$ resulting in the formation of CoQ_{10} and α-TOH (not shown). Second, $CoQ_{10}H^{\bullet}$ at the lipophilic/aqueous interface may deprotonate and the resulting $CoQ_{10}^{\bullet-}$ autoxidize to form the charged $O_2^{\bullet-}$ that escapes to the aqueous environment (Reaction 9.7). It is assumed that lipid peroxyl radicals (LOO$^{\bullet}$) and α-TO$^{\bullet}$ move freely within though do not readily escape from oxidizing lipoprotein particles.[57] L$^{\bullet}$, carbon-centered lipid radical; LOOH, lipid hydroperoxide.

explains the "lag-phase" observed during the commonly employed Cu^{2+}/LDL oxidation test system.[49] However, under low radical flux conditions α-TO$^{\bullet}$ is not "eliminated" so that chain-transfer (Reaction 9.2, Figure 9.1) predominates and hence α-TOH exhibits prooxidant activity. For Cu^{2+} as the oxidant, α-TOH switches from a prooxidant to an antioxidant at a Cu^{2+} to LDL ratio of $\approx$3.[28] With peroxynitrite added as a bolus, the switching point occurs at oxidant to LDL ratios of 100:1 to 200:1.[31] Whether vitamin E exhibits pro- or antioxidant activity is also determined by the reactivity of the oxidant.[17] For highly reactive oxidants (e.g., $^{\bullet}$OH), a lower radical flux is required to achieve a prooxidant activity when compared to less reactive oxidants (e.g., ROO$^{\bullet}$).[17] Thus, the point at which α-TOH switches from a pro- to an antioxidant is reached at a radical flux of $\approx$130 and 250 nM/min for $^{\bullet}$OH and ROO$^{\bullet}$, respectively.[17] Extensive studies have confirmed that TMP is relevant for oxidizing conditions that promote formation of free radicals. These include Cu^{2+}, human monocytes or macrophages cultured in the transition metal containing Ham's F-10 medium, 15-lipoxygenase, hydroxyl radical ($^{\bullet}$OH), peroxynitrite (either added as a bolus or delivered in a time-dependent manner by the simultaneous generation of $O_2^{\bullet-}$ and $^{\bullet}$NO), MPO-derived tyrosyl

radicals, myoglobin and horseradish peroxidase/H_2O_2.[15,17,18,25,28–31,61] In contrast, two-electron oxidants such as HOCl do not induce substantial lipid peroxidation.[63] However, among the oxidized amino acid adducts formed by treatment of LDL with HOCl are chloramines that break down to yield "secondary" radicals[64] that induce LDL lipid peroxidation via TMP.[65] Similar to HOCl, apo B (rather than lipids) is the major target for peroxynitrite-induced oxidation reactions.[31] However, peroxynitrite also induces one-electron reactions[66] and hence LDL lipid peroxidation via TMP.[31]

9.3 COANTIOXIDATION

The chain-transfer reaction of α-TO$^\bullet$ is kinetically unfavorable ($k_{TMP} \approx 0.05\ M^{-1}s^{-1}$ [15]) although thermodynamically possible.[67] As such, the prooxidant activity of α-TOH is effectively prevented by compounds that reduce α-TO$^\bullet$ and subsequently eliminate the radical from a lipoprotein particle.[32,59,68–70] Such compounds are termed coantioxidants.[32] Importantly, regeneration of α-TOH from α-TO$^\bullet$ alone does not equate with coantioxidation. It is the conversion of the *lipophilic* α-TO$^\bullet$ into harmless *aqueous* radical(s) or nonradical products (NRP) by the coantioxidant that prevents lipid peroxidation in lipoproteins.[32]

As indicated earlier, when α-TOH acts as a chain-breaking antioxidant up to one molecule of lipid hydroperoxide is formed for each molecule of vitamin E consumed.[36] In contrast, the TMP model predicts that the formation of lipid hydroperoxides is strongly suppressed in the presence of α-TOH *and* a co-antioxidant. Therefore, coantioxidants make α-TOH a more effective antioxidant for lipoprotein lipids.[32,59] A variety of lipophilic and aqueous compounds represent potential physiologically relevant coantioxidants as judged by their *in vitro* activity. These include $CoQ_{10}H_2$,[14,18] α-tocopheryl hydroquinone (α-TQH_2),[71] ascorbate,[15,55,69,72] and 3-hydroxyanthranilate.[69] The following will focus on $CoQ_{10}H_2$ as a coantioxidant.

9.3.1 $CoQ_{10}H_2$ Levels in Plasma Lipoproteins

Freshly and rapidly isolated plasma lipoproteins contain only small amounts of $CoQ_{10}H_2$ when compared to α-TOH. Thus, in unsupplemented human subjects, plasma contains ≈0.5 to 1.0 μM $CoQ_{10}H_2$ with ≈58, 26, and 16% being present in LDL, HDL, and VLDL/IDL, respectively.[73] Although accounting for a majority of $CoQ_{10}H_2$ present in plasma, isolated LDL contains, on average, one molecule of $CoQ_{10}H_2$ for every second lipoprotein particle.[16] Dietary supplementation with 100 to 300 mg/d of coenzyme Q increases the concentration of $CoQ_{10}H_2$ in plasma and all of its lipoproteins.[16] Maximal enrichment of plasma and LDL with $CoQ_{10}H_2$ appears to be achieved after 5 days continuous supplementation, after which time LDL's $CoQ_{10}H_2$ levels are increased four- to fivefold, i.e., from 0.5–0.8 to 2.0–3.0 $CoQ_{10}H_2$ molecules per LDL.[16,18] Interestingly, dietary enrichment with coenzyme Q_{10} may also result in an increase in LDL α-TOH levels.[16,18] The reasons for this remain to be elucidated.

In plasma lipoproteins more than 80% of the total coenzyme Q is present as $CoQ_{10}H_2$,[16] indicating that sufficient reducing potential is available to keep circulating coenzyme Q_{10} in the reduced, (co-) antioxidant active form. The recent study by Yamashita et al. shows that in healthy subjects, ≈95% of coenzyme Q is present as $CoQ_{10}H_2$[74] indicating that plasma lipoprotein levels of $CoQ_{10}H_2$ are potentially 15% greater than previously measured.[16,75] The redox status does not appear to be dependent on the absolute plasma concentration of coenzyme Q_{10}.[16] The reductive processes required to maintain lipoprotein associated $CoQ_{10}H_2$ are yet to be fully elucidated.[76,77] However, a vast majority of gavaged coenzyme Q in rats is detected as ubiquinol in mesenteric lymph, indicating that the intestinal tract represents one site where reduction takes place.[78] This could involve various intracellular enzymes such as DT-diaphorase,[79] a plasma membrane NADH-ubiquinone reductase,[80] and a cytosolic NADPH-ubiquinone reductase.[81]

9.3.2 $CoQ_{10}H_2$ is the First Lipophilic Antioxidant Consumed in *Ex Vivo* LDL Undergoing Oxidation

$CoQ_{10}H_2$ is the first antioxidant consumed when freshly isolated LDL is exposed to a vast array of oxidizing conditions including the radical oxidants mentioned above, as well as hypochlorite, singlet oxygen, peroxynitrite, and activated human phagocytes.[14,16,31,59,63,82–84] During oxidation of human plasma by aqueous oxidants, $CoQ_{10}H_2$ is consumed after ascorbate[18,31] (Figure 9.2A). In contrast, when plasma is oxidized with lipophilic ROO•, consumption of $CoQ_{10}H_2$ precedes that of ascorbate[85] (Figure 9.2B). In plasma or LDL undergoing oxidation, consumption of α-TOH and formation of oxidized lipids is markedly suppressed while $CoQ_{10}H_2$ is present[14,16,59] (Figure 9.2).

9.3.3 Enrichment of LDL with $CoQ_{10}H_2$ Inhibits LDL Lipid Peroxidation

To investigate a role for $CoQ_{10}H_2$ as an LDL (co-) antioxidant, we have previously compared the oxidizability of $CoQ_{10}H_2$-enriched LDL (isolated from human subjects supplemented for 5 to 11 days with 100 to 300 mg/day coenzyme Q) with the corresponding native, nonenriched LDL. Oxidizing conditions employed in these studies included ROO•,[16] 15-lipoxygenase,[84] peroxynitrite,[31] the transition-metal containing Ham's F-10 medium,[18] or Cu^{2+} (S. R. Thomas, J. Neuzil, and R. Stocker, unpublished observations). For all oxidizing conditions tested, $CoQ_{10}H_2$-enriched LDL was more resistant to lipid peroxidation than the native LDL. Figures 9.3 and 9.4 show the data for LDL exposed to 15-lipoxygenase[84] or the peroxynitrite generator 3-morpholinosydnonimine (SIN-1),[31]

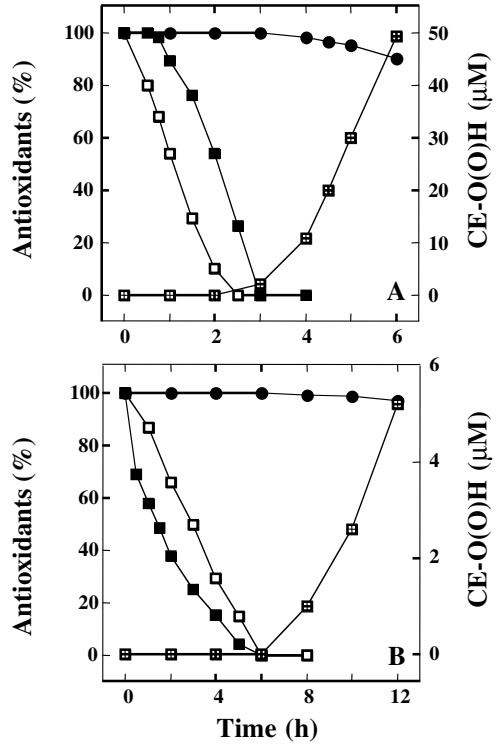

FIGURE 9.2 The order of $CoQ_{10}H_2$, and ascorbate consumption in human plasma is dependent on whether the radicals are lipophilic or aqueous in nature. Human plasma was exposed to either 10 mM AAPH (A, aqueous ROO•) or 2 mM AMVN (B, lipophilic ROO•) and incubated at 37°C. At the indicated times, aliquots were analyzed for ascorbate (.), $CoQ_{10}H_2$ (■), α-TOH (●), and CE-O(○)H (hatched squares). 100% values for the antioxidants were 48 μM ascorbate, 0.8 μM $CoQ_{10}H_2$, and 18 μM α-TOH.

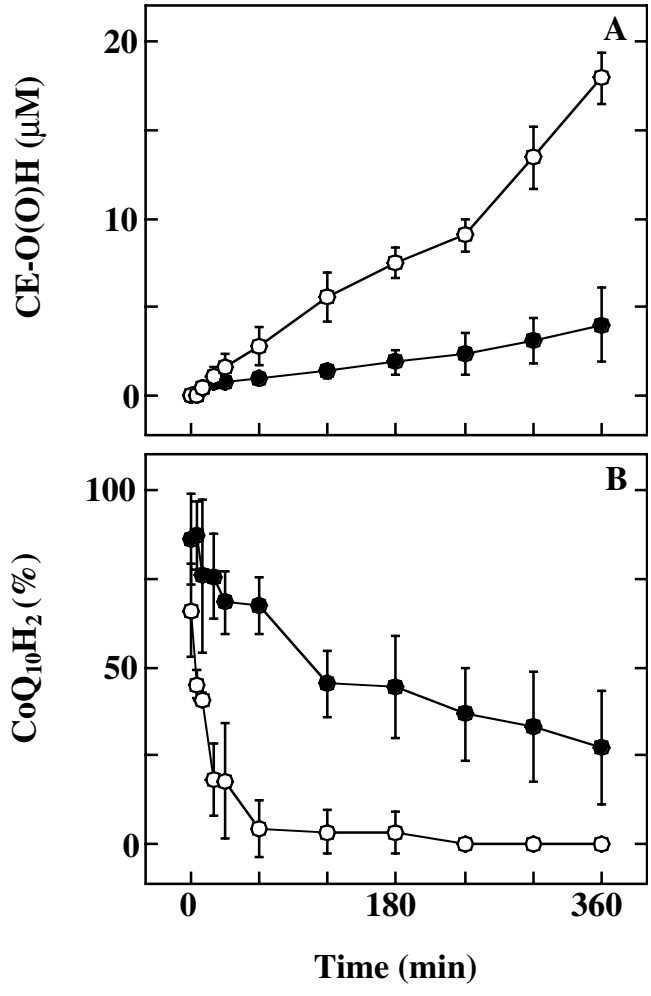

FIGURE 9.3 Dietary supplementation with coenzyme Q protects LDL lipids against oxidation induced by 15-lipoxygenase. *In vivo* $CoQ_{10}H_2$-enriched (closed symbols) or native (open symbols) LDL (0.86 ± 0.21 μM ApoB) was exposed to recombinant human 15-lipoxygenase (0.3 μM) and incubated at 37°C. At the indicated times, LDL aliquots were removed and analyzed for CE-O(O)H (A) and $CoQ_{10}H_2$ (B). 100% values for $CoQ_{10}H_2$ were 0.4 μM ± 0.2 and 2.3 μM ± 1.9 for native and $CoQ_{10}H_2$-enriched LDL, respectively. Results are the mean ±SD of 3 independent experiments using LDL from 3 different donors. Published with permission from [84].

respectively. Importantly, studies with Ham's F-10 medium as the oxidant have demonstrated that dietary coenrichment of LDL with $CoQ_{10}H_2$ and α-TOH prevents the prooxidant effect seen with enrichment with α-TOH alone[18] (Figure 9.5). The ability of $CoQ_{10}H_2$ to inhibit the prooxidant activity of α-TOH is consistent with $CoQ_{10}H_2$ representing an effective lipophilic coantioxidant.

A role for $CoQ_{10}H_2$ as an important antioxidant for LDL lipids is also supported by studies by Kontush et al.[19] and Tribble et al.[86] who reported that the content of $CoQ_{10}H_2$ correlates negatively with the susceptibility of LDL to the initial stages of Cu^{2+}-induced oxidation. In contrast, a recent study[87] has reported no protective effect of dietary coenzyme Q supplementation on *ex vivo* LDL oxidation. However, only total coenzyme Q content was measured in this study and no special care was taken to preserve $CoQ_{10}H_2$ in the lipoprotein and elevated levels of $CoQ_{10}H_2$ at the initiation of the oxidation experiments were not confirmed. $CoQ_{10}H_2$ is highly susceptible to autoxidation (during

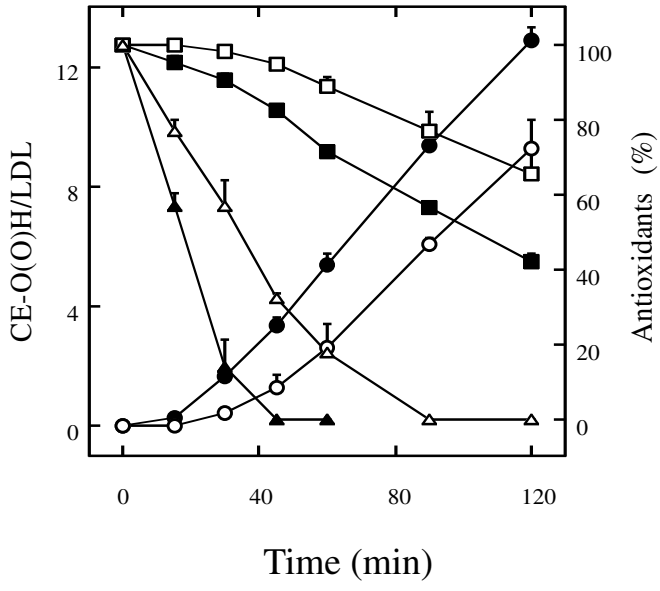

FIGURE 9.4 Dietary supplementation with coenzyme Q protects LDL lipids against oxidation induced by peroxynitrite. Dietary $CoQ_{10}H_2$-enriched (open symbols) or native (closed symbols) LDL (0.5 mg protein/mL) was exposed to the peroxynitrite generator, SIN-1 (40 μM). At the indicated times, LDL aliquots were removed and analyzed for $CoQ_{10}H_2$ (triangles), α-TOH (squares) and CE-O(O)H (circles). 100% values for $CoQ_{10}H_2$ were 0.6 μM $\pm$ 0.1 and 2.4 μM $\pm$ 0.5 and for α-TOH were 8.3 μM $\pm$ 0.2 and 10.6 μM $\pm$ 0.2 for native and $CoQ_{10}H_2$-enriched LDL, respectively. Results are the mean $\pm$ SEM of 3 independent experiments using LDL from 3 different donors. Published with permission from [31].

plasma storage and/or subsequent LDL isolation) and hence can be lost unless stringent precautions are taken.[16,18,88] Therefore, it is pertinent that studies examining a potential antioxidant function of $CoQ_{10}H_2$ establish its presence prior to oxidation experiments.

At present there is a lack of knowledge as to whether $CoQ_{10}H_2$ acts as an effective antioxidant *in vivo*. To answer this issue, future studies may consider assessing the effect of coenzyme Q supplementation on plasma and tissue levels of isoprostanoids, a class of nonenzymatic oxidation products of arachidonic acid.[89]

9.3.4 Mechanism of $CoQ_{10}H_2$ Antioxidant Action for LDL Lipids

An important issue is how small levels of $CoQ_{10}H_2$ provide significant antioxidant protection for LDL lipids when the lipoprotein is exposed to a variety of different oxidizing conditions. We have previously proposed[15,18,56,60,62] that $CoQ_{10}H_2$ scavenges α-TO$^\bullet$ rather than oxidation-initiating radicals, and as such represents a lipophilic coantioxidant. In support of this mechanism, $CoQ_{10}H_2$ is consumed before α-TOH, despite α-TOH and $CoQ_{10}H_2$ exhibiting similar rate constants for the scavenging of peroxyl radicals and α-TOH being present in LDL at a 5 to 10 times higher concentration. Also, the hydrophobic side chain of $CoQ_{10}H_2$ likely reduces its mobility within the lipoprotein particle and therefore decreases its access to radicals when compared to the more polar α-TOH. Therefore, α-TOH would be expected to outcompete $CoQ_{10}H_2$ with respect to scavenging radicals interacting with or present in LDL, resulting in the formation of α-TO$^\bullet$. Consistent with this notion, $CoQ_{10}H_2$ consumption is independent of the rate of radical generation (R_g) whereas the rate of α-TOH consumption increases with increasing R_g.[71] Also, $CoQ_{10}H_2$ does not affect the rate of α-TOH-mediated reduction of Cu^{2+} and hence the rate of formation of α-TO$^\bullet$ in LDL exposed to this

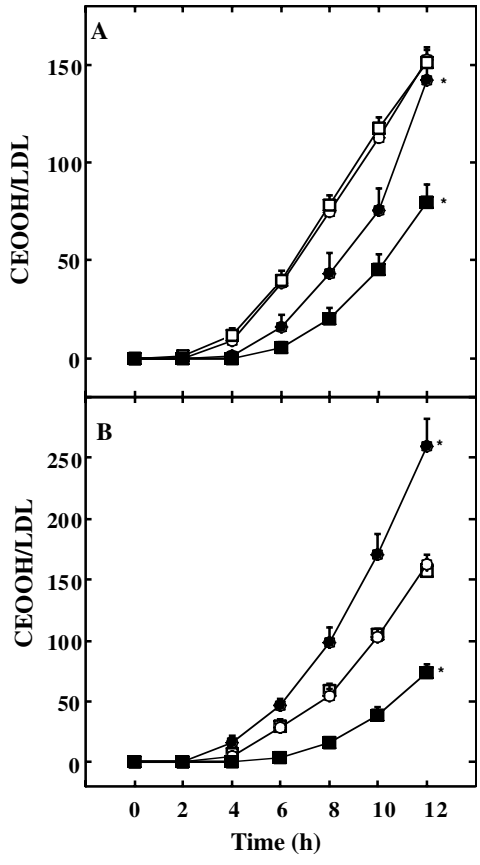

FIGURE 9.5 Dietary supplementation with coenzyme Q efficiently protects LDL against the prooxidant effect of α-TOH supplementation alone. LDL was incubated in Ham's F-10 medium at a final concentration of 0.1 to 0.2 mg protein/mL. (A) Enriched LDL samples were obtained from subjects supplemented with coenzyme Q_{10} for 6 hours ($n = 3$) (●) or 5 days ($n = 8$) (■), and their oxidation was compared with the corresponding native LDL isolated from nonsupplemented plasma after 6 hours (○) or 5 days (□) of storage. (B) The oxidation of enriched LDL samples, from 8 subjects supplemented initially with α-TOH alone for 6 hours (●) and then cosupplemented with α-TOH and coenzyme Q for 5 days (■) was compared with that of native LDL isolated from plasma taken prior to supplementation and stored for 6 hours (○) or 5 days (□). The 100% values for α-TOH were 1.8 ± 0.6 μmol/L for control LDL,3.25 ± 0.7 μmol/L for α-TOH-enriched LDL, and 4.7 ± 0.8 μmol/L for coenriched LDL. The results shown represent means ±SEM of $n = 8$ carried out in duplicate. For statistical analysis, repeated measures ANOVA comparing lines of CE-OOH values of supplemented LDL samples and those of the corresponding native LDL samples were carried out. *Significant difference ($P < 0.01$). Published with permission from [18].

transition metal,[90] yet $CoQ_{10}H_2$ inhibits LDL lipid peroxidation induced by this oxidant.[18] Also, $CoQ_{10}H_2$ can reduce α-TO$^\bullet$ directly.[91] Finally, enrichment of LDL with $CoQ_{10}H_2$ inhibits LDL lipid peroxidation by oxidants that induce the formation of α-TO$^\bullet$ in LDL and oxidize lipoprotein lipids via TMP.[15,18,31,84] Together, the above indicate that $CoQ_{10}H_2$ protects LDL lipids from oxidation most likely by acting as a coantioxidant (Reaction 9.6, Figure 9.1).

Scavenging of α-TO$^\bullet$ by $CoQ_{10}H_2$ results in the regeneration of α-TOH and the formation of the $CoQ_{10}H^\bullet$ (Reaction 9.6, Figure 9.1). The pK_a of ubisemiquinone radical is 6.5, so that at physiological pH and at the lipid/aqueous interface, it will be present as $CoQ_{10}^{\bullet-}$. In the nonpolar and aprotic lipophilic environment of the interior of LDL, $CoQ_{10}H^\bullet$ will predominate. $CoQ_{10}^{\bullet-}$ in

LDL could conceivably scavenge a second α-TO$^{\bullet}$ (not shown in Figure 9.1). This reaction is thermodynamically more favorable than that of $CoQ_{10}H_2$ with α-TO$^{\bullet}$ as the one-electron redox potential of the $CoQ_{10}/CoQ_{10}^{\bullet-}$ couple is lower (−36 mV) than that of the $CoQ_{10}^{\bullet-}/CoQ_{10}H_2$ couple (200 mV).[92] Indeed, this reaction has been experimentally verified in organic solvents.[93] However, for this reaction to occur, a second α-TO$^{\bullet}$ would need to be present in oxidizing LDL, whereas TMP proposes that only one α-TO$^{\bullet}$ is present.[15] Therefore, a reaction of $CoQ_{10}^{\bullet-}$ (or $CoQ_{10}H^{\bullet}$) and α-TO$^{\bullet}$ is not likely important in an oxidizing LDL particle. Also, $CoQ_{10}H^{\bullet}$ is unlikely to effectively reduce α-TO$^{\bullet}$ as the one-electron redox potential is likely to be similar to that of α-TO$^{\bullet}$.

Alternatively, the relatively polar benzoquinone moiety of coenzyme Q_{10} may gain access to the aqueous interface of LDL. In this case, $CoQ_{10}H^{\bullet}$ will likely deprotonate and the resulting $CoQ_{10}^{\bullet-}$ autoxidize to yield $O_2^{\bullet-}$ (Reaction 9.7, Figure 9.1).[44] Whether $CoQ_{10}H^{\bullet}$ autoxidizes in LDL's interior is not known, although in the nonpolar, aprotic interior of liposomes, this reaction is suggested to be unlikely.[44] In any case, the resulting $^{\bullet}$OOH would be expected to reinitiate lipid peroxidation,[47] whereas the presence of $CoQ_{10}H_2$ in LDL effectively suppresses lipid peroxidation. This suggests that formation of $O_2^{\bullet-}$ is more likely. Whether the putative $O_2^{\bullet-}$ formed dismutates (into hydrogen peroxide and oxygen) or reduces a second molecule of α-TO$^{\bullet}$ is not known.[56]

In summary, each molecule of $CoQ_{10}H_2$ scavenges at least 1 radical chain-propagating α-TO$^{\bullet}$ and thereby terminates 1 radical chain reaction. As LDL lipid peroxidation proceeds as a radical chain reaction in the presence of α-TOH with chain-lengths of up to 25 reported, $CoQ_{10}H_2$ may cause the rate of the peroxidation to decrease by up to 25 times. Also, the degree of inhibition decreases with the *square root* of the concentration of the "coantioxidant."[15,32] Therefore, coantioxidant action of $CoQ_{10}H_2$ (Reactions 9.6–9.7, Figure 9.1) can readily explain why even small amounts of $CoQ_{10}H_2$ effectively inhibits LDL lipid peroxidation induced by a mild oxidative stress. As LDL in unsupplemented humans contains on average <1 $CoQ_{10}H_2$ molecule per particle it may be particularly relevant that the $CoQ_{10}H_2$ content can be increased to an average number to >1 as this would be expected to increase the resistance of all lipoprotein particles towards lipid peroxidation. The efficiency of $CoQ_{10}H_2$ as a LDL coantioxidant may be further enhanced in the presence of effective reductive processes that maintain coenzyme Q in the reduced state.

A recent study has demonstrated that α-TQH_2, the two electron reduction product of α-tocopheryl quinone, can reduce CoQ_{10} to $CoQ_{10}H_2$ and α-TO$^{\bullet}$ to α-TOH in lipoproteins.[71] Interestingly, reduction of CoQ_{10} by α-TQH_2 was observed only in intact lipoproteins, whereas it did not occur in organic solution or lipid emulsions.[71] Importantly, α-TQH_2 is stable when incorporated into lipoproteins, is consumed before $CoQ_{10}H_2$ and α-TOH in oxidizing LDL, and therefore represents a most efficient antioxidant in lipoproteins.[71] This contrasts with results obtained from experiments using methyl linoleate in organic phase or aqueous dispersions that demonstrate that α-TQH_2 is prone to autoxidation and that α-TOH represents a more potent antioxidant than α-TQH_2 and $CoQ_{10}H_2$.[94] The differences described above highlight the need for caution in extrapolating results obtained from experiments performed in artificial systems to biomembranes and lipoproteins. Although yet to be substantiated, α-TQH_2 may be present *in vivo* at sites of inflammation such as atherosclerotic lesions where α-TOH oxidation to α-tocopheryl quinone occurs[95] and (enzymatic) reducing system(s) may be present that convert α-tocopheryl to quinone α-TQH_2.[96] This suggests that α-TOH may provide a potential "reservoir" for a coantioxidant that spares vitamin E from further consumption.[97] α-TQH_2 can be detected in human plasma lipoproteins after dietary intake of α-tocopheryl quinone indicating that mechanisms exist *in vivo* capable of reducing the two-electron oxidation product of α-TOH.[97] Also, a recent study suggested that oral α-tocopheryl quinone may be converted to α-TOH.[98]

Dihydrolipoic acid also maintains $CoQ_{10}H_2$ in the reduced state by two-electron reduction of CoQ_{10} or one-electron reduction of $CoQ_{10}^{\bullet-}$.[99] However, whether dihydrolipoic acid is capable of maintaining lipoprotein-associated $CoQ_{10}H_2$ remains to be demonstrated. It is important to note that in this context, under normal conditions, dihydrolipoic acid is not present in plasma.[100]

9.4 COENZYME Q AND ATHEROSCLEROSIS

9.4.1 Levels of α-TOH and $CoQ_{10}H_2$ in Plasma of Patients with Cardiovascular Disease

As $CoQ_{10}H_2$ is the first lipoprotein-associated antioxidant consumed when plasma is exposed to various oxidizing conditions, it has been proposed that the plasma coenzyme Q_{10} redox status [i.e., $CoQ_{10}H_2/(CoQ_{10}H_2 + CoQ_{10})$] may represent a useful diagnostic indicator for *in vivo* lipoprotein oxidation and oxidative stress.[74] A number of studies have compared the levels of $CoQ_{10}H_2$ in the plasma of patients with advanced atherosclerosis with that of age-matched controls. On average, plasma $CoQ_{10}H_2$ and the coenzyme Q redox status are slightly lower, and the levels of α-TOH slightly higher in patients exhibiting clinical indices of coronary artery disease.[101,102] Importantly, these changes in plasma antioxidant levels were not significant.[101] There also appears to be no significant difference in the levels of antioxidants in isolated LDL from patients and controls, and hydroperoxides of cholesteryl esters were undetectable (detection limit 10 nM) in both patients and controls.[101] Thus, plasma and LDL levels of lipophilic antioxidants, including $CoQ_{10}H_2$, appear only partially oxidized even in subjects with severe atherosclerosis.[101,102] Overall, this is consistent with the assumption that oxidized lipoproteins detected in atherosclerotic lesions are derived primarily from oxidation reactions occurring within the arterial wall.

In contrast to coronary artery disease patients, a significant decrease in the plasma coenzyme Q_{10} redox status has been reported for patients with hyperlipidemia, liver disease (hepatitis, hepatoma, cirrhosis), or treated with percutaneous transluminal coronary angioplasty.[75,103–105]

9.4.2 Is TMP Relevant for *In Vivo* Lipid Oxidation during Atherosclerosis?

Whether TMP of lipoprotein lipids occurs *in vivo* is difficult to prove. However, various lines of evidence suggest that lipid oxidation detected in atherosclerotic lesions occurs in the presence of α-TOH, and in part, via TMP.[23] Thus, oxidants thought to promote LDL oxidation *in vivo* promote LDL lipid peroxidation *in vitro* via TMP. Also, substantial amounts of oxidized lipids coexist with normal levels of α-TOH.[95,106] In addition, 60 to 70% of cholesteryl linoleate alcohols (Ch 18:2-OH), the predominant lipid oxidation products present in human atherosclerotic lesions[95,107] are present as the nonenzymatic and thermodynamically unfavorable *cis*, *trans*-isomers.[23] This indicates that they were formed in the presence of α-TOH.[108,109] Finally, where examined, coantioxidants inhibit aortic lipoprotein lipid peroxidation in the aortas of atherosclerosis prone animals.[12,110] Together, these findings are consistent with, though are not conclusive proof that oxidation of lipoprotein lipids in the atherosclerotic lesions occurs primarily via TMP. The findings are largely inconsistent with the common assumption that substantial LDL lipid oxidation requires depletion of α-TOH.[49] Given the important consequences, more work is required to establish whether, and if so to what extent, lipoprotein lipid oxidation occurs in the absence or presence of vitamin E.

9.4.3 Coenzyme Q_{10} Content in Atherosclerotic Lesions

With respect to inhibition of *in vivo* lipid peroxidation during atherogenesis, $CoQ_{10}H_2$ is a prime candidate as an *in vivo* (co-) antioxidant for a number of reasons. First, unlike aqueous coantioxidants, $CoQ_{10}H_2$ is incorporated in lipoproteins, and as such can exhibit site-specific coantioxidation. Second, $CoQ_{10}H_2$ inhibits LDL lipid peroxidation induced by both strong and mild oxidizing conditions[18] (S. R. Thomas and R. Stocker, unpublished observations). Third, $CoQ_{10}H_2$ is a first line of lipophilic antioxidant defense in lipoproteins and plasma against physiologically relevant oxidants. Fourth, supplementation with coenzyme Q increases the lipoprotein's content of $CoQ_{10}H_2$ from <1 to >1 molecule per particle and this increases the oxidation resistance (see above). Finally, coenzyme Q

appears to be a safe dietary supplement with no reported side effects, although long-term studies with relatively high doses (100 to 200 mg/day) remain to be carried out.

In atherosclerotic lesions, $CoQ_{10}H_2$ has not been detected despite normal concentrations of total coenzyme Q being present.[95] However, any remaining $CoQ_{10}H_2$ may have autoxidized during sample preparation. If coenzyme Q is present predominantly as CoQ_{10} in the arterial wall, then this would increase the susceptibility of lipoproteins to oxidation. A recent study[111] using apolipoprotein E knockout mice fed a high fat diet has reported that aortic coenzyme Q content decreases proportionally to α-TOH as the atherosclerotic lesions develop. This, too, may increase the susceptibility of lipoprotein lipids to oxidation.[18] However, dietary supplementation of these mice with coenzyme Q_{10} can increase aortic coenzyme Q up to 10 times thereby rectifying the imbalance with α-TOH seen in unsupplemented mice.[62]

9.4.4 Does Coenzyme Q_{10} Supplementation Attenuate Experimental Atherosclerosis?

Recent studies have shown that supplementation of atherosclerosis susceptible apo-E gene knockout mice with coenzyme Q_{10} results in substantially increased plasma and aortic levels of $CoQ_{10}H_2$.[62] Importantly, this was associated with a decrease in the concentration of aortic lipid hydroperoxides and the extent of atherosclerosis in the aorta (P. K. Witting, K. Pettersson, R. Stocker, in preparation). In light of the oxidation theory, it might be assumed that the ability of $CoQ_{10}H_2$ to inhibit aortic lipid oxidation is the primary reason for its antiatherosclerotic activity. However, a recent study has shown that aortic lipid oxidation and atherosclerosis can be dissociated[12] and antioxidants may inhibit atherosclerosis independent of LDL oxidation.[11,24] Further studies are required to validate the antiatherogenic activity of coenzyme Q.

9.5 CONCLUSION

TMP represents a valid model to explain the molecular actions of vitamin E in controlling lipoprotein lipid peroxidation and explains why the antioxidant activity of vitamin E is enhanced or even dependent on the presence of coantioxidants, of which $CoQ_{10}H_2$ may represent the most important physiologically relevant example. There is some evidence supporting a participation of TMP for *in vivo* lipoprotein lipid oxidation in human atherosclerotic lesions. Where tested, coantioxidants inhibit *in vitro*[32,59,70] and *in vivo* lipoprotein lipid peroxidation[12,110] and $CoQ_{10}H_2$ represents the first line of lipophilic coantioxidant defense. Although low levels of $CoQ_{10}H_2$ are present in plasma lipoproteins, dietary supplementation with coenzyme Q_{10} increases both the content of $CoQ_{10}H_2$ in lipoproteins and their resistance to lipid peroxidation. Although implicated, to date there is no conclusive proof that LDL lipid oxidation causes atherosclerosis, and that vitamin E supplementation alone inhibits atherosclerosis in humans and animals. These inconclusive results are not surprising considering that atherosclerosis is a complex disease involving multifactorial causes. If LDL lipid oxidation represents an important cause of disease, then supplementation with vitamin E plus coantioxidants, rather than vitamin E alone, represents a logical antiatherogenic strategy. Therefore, future human and animal intervention studies investigating the ability of coenzyme Q supplementation, either alone or in combination with vitamin E, are warranted.

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10 Biochemical and Pharmacological Properties of Coenzyme Q Analogs

Alvaro Mordente, Elisabetta Meucci, Giuseppe Ettore Martorana, Giorgio Minotti, and Bruno Giardina

CONTENTS

10.1 CHEMISTRY AND BIOCHEMISTRY OF COENZYME Q ANALOGS

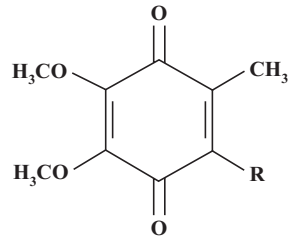
Mammalian membranes contain coenzyme Q_n (CoQ_n) homologues with long isoprenoid chains (Q_{10} for humans and Q_9 and Q_{10} for rodents), which are the major nonprotein components of the bioenergetic system of mitochondria.[1] Aging and several degenerative diseases are associated with a decline in the normal levels of CoQ_{10}, hence, CoQ_n supplementation has been introduced as a potential therapy to ameliorate energy deficiencies associated with these pathophysiological states.[1–4] Nonetheless, the beneficial effects of CoQ_n supplementation cannot be altogether separated from its well-known antioxidant potential (see section 10.2).

The role of CoQ_{10} is to shuttle electrons from complex I or complex II to complex III. Myopathies, encephalomyopathies, and several neurodegenerative diseases are characterized by malfunctioning of complex I.[5,6] Short chain coenzyme Q homologues (from Q_1 to Q_3) and analogs (pentylubiquinone, decylubiquinone, and idebenone) are a class of artificial quinones which are commonly employed *in vitro* as substitutes for the natural CoQ_{10} in order to evaluate whether they can restore the appropriate function of complex I or, more generally, to assess how specifically they can associate with such complex.[7–9] In fact, the hydrophobic side chain makes CoQ_{10} water-insoluble and precludes its use as a substrate for NADH:CoQ oxidoreductase in aqueous media. The alkyl tail, which facilitates lateral diffusion in biomembranes, is not critical to the redox properties of the polar benzoquinone ring, which is the basic functional group of the molecule. However, the role of the side chain is not simply to modulate the hydrophobicity of the molecule, but rather to participate in determining the specificity and kinetics of substrate-enzyme interaction through molecular recognition by the quinone binding site of mitochondrial complexes.[7–9] Therefore, having coenzyme Q homologues and analogs with different alkyl tails allows the performance of structure-activity studies to establish requirements for association of such quinones with the mitochondrial binding sites.

Chemical structures of the quinones discussed in this review are shown in Table 10.1. CoQ_1, CoQ_2, and CoQ_3 have a side chain composed of one, two, and three isoprenoid units, respectively,

0-8493-8732-9/00/$0.00+$.50

TABLE 10.1
Structure and Hydrophobicity of Short Chain Coenzyme Q Analogs



Compound	R (C_6 Substituent)	Hydrophobicity (log P)[a]
CoQ_1	CH_2–CH=C(CH_3)–CH_3	2.65
PB	CH_2–(CH_2)$_3$–CH_3	3.70
IDB	CH_2–(CH_2)$_8$–CH_2OH	n.d.[b]
CoQ_2	(CH_2–CH=C(CH_3)–CH_2)$_2$–H	5.10
DB	CH_2–(CH_2)$_8$–CH_3	7.20
CoQ_3	(CH_2–CH=C(CH_3)–CH_2)$_3$–H	7.70

[a] Partition coefficients (cyclohexane/water) according to [7].
[b] Not determined. IDB hydrophobicity is considered intermediate between PB and CoQ_2.[8]

whereas pentylubiquinone (PB), decylubiquinone (DB), and idebenone (IDB) contain a saturated pentyl, decyl, and hydroxydecyl tail, respectively. Only CoQ_1, PB, and DB are efficient substrates of NADH:CoQ oxidoreductase with reduction rates comparable to that calculated for endogenous CoQ_{10}.[7–9] On the contrary, CoQ_2, CoQ_3, and IDB are poor electron acceptors for complex I activity.[7–9] Indeed, they are incompletely reduced by NADH:CoQ oxidoreductase and form unstable semiquinone intermediates that eventually inhibit complex I, presumably by forming oxygen radicals through redox coupling of the semiquinone with molecular oxygen.[7–9] IDB is a stronger inhibitor of NADH:CoQ oxidoreductase and of the complex-generated membrane potential than CoQ_2.[8] Since CoQ_2 and IDB, like the other short chain quinones, are efficient substrates for both complexes II and III, and inhibition of the overall NADH oxidase activity basically derives from their strong inhibition of complex I. A direct consequence of these findings is that CoQ_2, CoQ_3, and IDB should fail in restoring energy deficiencies under pathological conditions, potentially making their therapeutic use of questionable efficacy, if not actually harmful.[8] In the case of IDB, such concerns have not been supported by clinical evidence (see section 10.3).

10.2 ANTIOXIDANT PROPERTIES OF COENZYME Q ANALOGS

Many experimental data strongly suggest that short chain quinones, in their reduced form, can function as antioxidants by inhibiting lipid peroxidation in model systems[10–14] and in biological membranes[10,15–19] (Table 10.2). Lipid peroxidation proceeds by a classic chain reaction mechanism that includes the three discrete phases of initiation, propagation, and termination (Figure 10.1). Initiation occurs when an "oxidant" of sufficient chemical reactivity abstracts a hydrogen atom from a polyunsaturated fatty acid (LH) producing an alkyl radical ($L^{\bullet}$) which, in turn, yields a lipid peroxyl radical ($LOO^{\bullet}$) by a diffusion-limited reaction with O_2. Once formed, $LOO^{\bullet}$ "propagates"

TABLE 10.2
Antioxidant Effects of Short Chain CoQ Analogs on Lipid Peroxidation

Short Chain Quinone	Oxidant System	System Tested
CoQ_1H_2–CoQ_3 H_2	LMW iron	Brain synaptosomes and mitocondria,[17] liver microsomes[18]
CoQ_3H_2	Peroxyl radical	Lipid vescicles[12]
CoQ_3H_2	Peroxyl radical	Liposomes[11]
CoQ_1H_2–CoQ_2H_2	FerrylMb	Fatty acids[13]
$IDBH_2$	FerrylMb, FerrylHb, LMW iron, Peroxyl radical	Fatty acids, liposomes, liver microsomes[14]
$IDBH_2$	LMW iron	Brain homogenate,[15] brain mitochondria,[16] brain synaptosomes[19]
$IDBH_2$	LMW iron	Fatty acids, brain and liver microsomes[10]

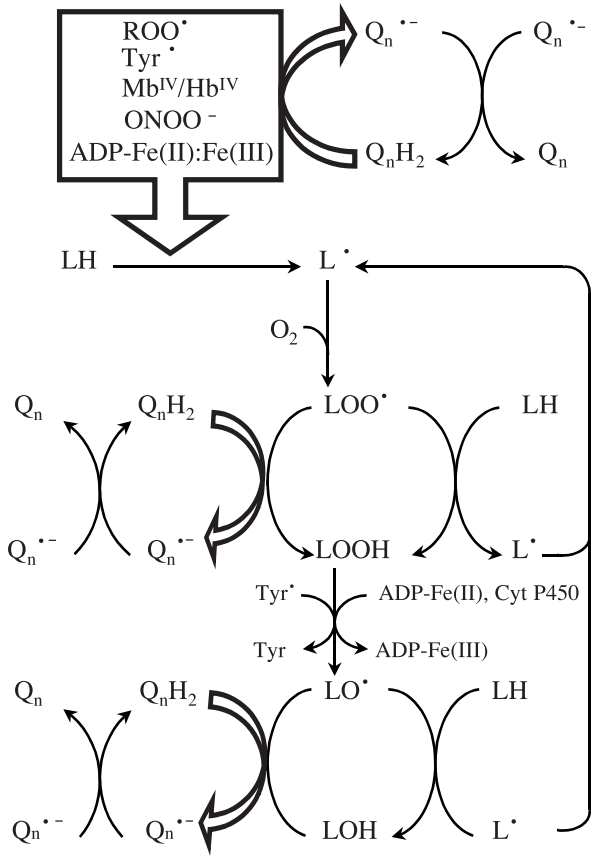


FIGURE 10.1 Protective effect of short chain coenzyme Q analogs against the initiation and propagation phase of lipid peroxidation. LH, polyunsaturated fatty acid moiety of a phospholipid; $L^{\bullet}$, carbon-centered fatty acid radical; $LOO^{\bullet}$, lipid peroxyl radical; LOOH, lipid hydroperoxide; $LO^{\bullet}$, lipid alkoxyl radical; LOH, lipid alcohol; Q_nH_2, short chain Coenzyme Q analogs, hydroquinone form; $Q_n^{\bullet -}$, semiquinone form; $ROO^{\bullet}$, peroxyl radical; $Tyr^{\bullet}$, tyrosyl radical; Hb^{IV}, ferrylhemoglobin; Mb^{IV}, ferrylmioglobin; $ONOO^{-}$, peroxynitrite.

a chain reaction by abstracting a *bis*-allylic hydrogen atom from nearby alkyl groups, generating a lipid hydroperoxide (LOOH) and another L$^{\bullet}$. These propagation cycles will repeat until oxygen and lipid substrates are available and will be terminated by reactions that deplete the system of propagating radicals.

Peroxyl radicals,[20] tyrosyl radicals,[21] activated hemoproteins,[22] peroxynitrite,[23] and redox-active low molecular weight (LMW) iron[24] are the best known initiators of lipid peroxidation. Short chain coenzyme Q analogs can interfere with the reactivity of these species. Peroxyl radicals (ROO$^{\bullet}$) are probably the O_2-derived oxidants attaining the highest steady-state concentration in biological systems, as any carbon-centered radical that is generated will add O_2 at a diffusion-controlled rate. A constant flow of peroxyl radicals can be produced *in vitro* by thermal decomposition of water-soluble (AAPH) and lipid-soluble (AMVN) azo-compounds.[25] $IDBH_2$[14] and CoQ_1H_2[26] protect biomolecules irrespective of the environments in which they are placed. Hydroquinones, indeed, prolong the lag phase that precedes peroxyl radical-dependent oxidation of water-soluble R-phycoerythrin or slow down the peroxidation of liposome-incorporated cis-parinaric acid,[14,26] suggesting that $IDBH_2$ and CoQ_1H_2 may intercept peroxyl radicals in both aqueous phases and lipid-water interfaces. In aqueous environments, $IDBH_2$ scavenges two peroxyl radicals per mole, showing an efficiency comparable to that of Trolox, a hydrophilic vitamin E analog. Under comparable conditions, $CoQ_{10}H_2$ and α-tocopherol are ineffective.[14,27] In hydrophobic phases, $IDBH_2$ is a slightly but significantly stronger radical scavenger than α-tocopherol.[14] Once embedded in large unilamellar vesicles or in liposomes, CoQ_3H_2, too, is capable of scavenging peroxyl radicals generated by AMVN or AAPH.[11,12]

Tyrosyl radicals (Tyr$^{\bullet}$) are known to promote lipid peroxidation either by abstracting hydrogen from polyunsaturated fatty acid or by decomposing endogenous hydroperoxides.[21,28] CoQ_1H_2, CoQ_2H_2,[13] and $IDBH_2$[14] can quench tyrosyl radicals, as evidenced by their ability to delay dityrosine formation after UV irradiation of tyrosine solution.

The hypervalent states of hemoproteins are strong oxidants that induce oxidation, peroxidation, and epoxidation of various biomolecules *in vitro*.[22] In the presence of H_2O_2, Mb^{III} as well as Hb^{III} undergo a two-electron oxidation process yielding a transient protein radical, probably a tyrosyl radical, plus a long lived oxoferryl moiety (Mb^{IV}), which can be detected spectrophotometrically as changes in the visible and Soret regions.[29,30] In particular, Mb^{IV}, but not the protein radical, has been found to promote the peroxidation of fatty acids, membranes, and lipoproteins.[31] Antioxidants that donate electrons to the oxoferryl moiety protect against oxidative damage.[22] CoQ_1H_2 and CoQ_2H_2[13] or $IDBH_2$[14] irreversibly and dose-dependently reduce Mb^{IV} to Mb^{III}. The reduction of Mb^{IV} to Mb^{III} is paralleled by the oxidation of short chain hydroquinones with a stoichiometry averaging unity.[13,14] By this mechanism, hydroquinones prevent the peroxidation of arachidonic acid (Figure 10.2) as well as of cis-parinaric acid incorporated in liposomes.[14] Moreover, CoQ_1H_2 protects creatine kinase from inactivation by ferrylmyoglobin, indicating that proteins subjected to oxidative stress can be shielded by short chain hydroquinones, too.[32] Similar results are obtained whenever myoglobin is replaced by hemoglobin.

Peroxynitrite ($ONOO^-$), a powerful oxidant that damages many cellular components,[33] is the product of the nearly diffusion-limited reaction between superoxide anion ($O_2^{\bullet -}$) and nitric oxide ($^{\bullet}NO$). Peroxynitrite can be produced *in vitro* by the spontaneous decomposition of 3-morpholinosydnonimine (SIN-1) at neutral pH and its formation can be detected by oxidation of dihydrorhodamine 123.[34] CoQ_1H_2 and CoQ_2H_2[26] and $IDBH_2$[14] can scavenge ONOO, thus inhibiting oxidative damage mediated by this species. Partially reduced species of dioxygen such as $O_2^{\bullet -}$ and H_2O_2 are not particularly reactive per se, but may become cytotoxic upon secondary reactions with other species, generating $ONOO^-$, Mb^{IV}, or Hb^{IV} and tyrosyl radicals (see above). Short chain hydroquinones (CoQ_1H_2, CoQ_2H_2, $IDBH_2$) and their respective oxidized forms lack reactivity with $O_2^{\bullet -}$ and H_2O_2,[13,14] but readily interact with those reactive species that would function as secondary and more potent mediators of oxidant damage, thereby mitigating the toxicity of $O_2^{\bullet -}$ and H_2O_2. One additional mechanism of toxicity by $O_2^{\bullet -}$ and H_2O_2 appears to involve the ability of these species

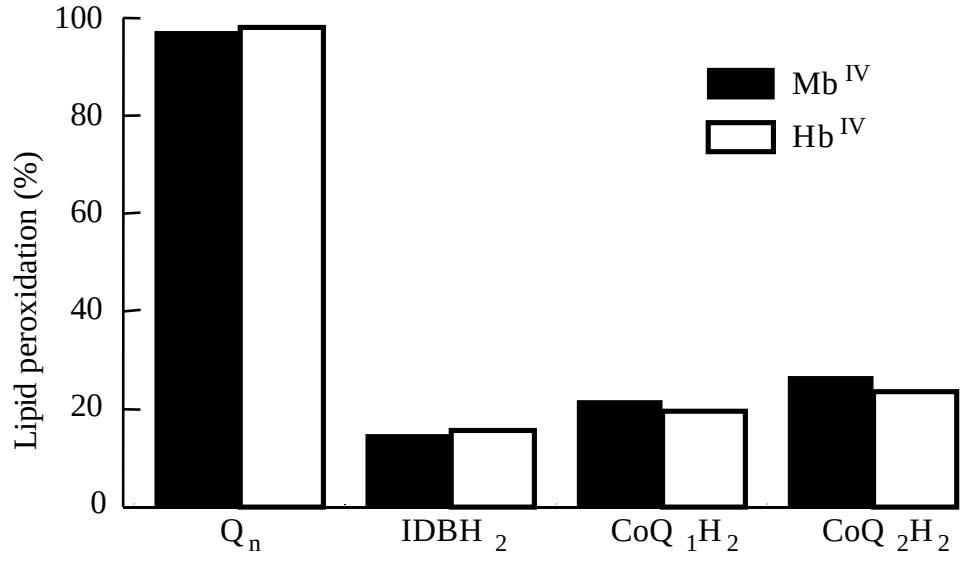


FIGURE 10.2 Effect of short chain coenzyme Q analogs on ferrylhemoprotein-dependent lipid peroxidation. Q_n, short chain coenzyme Q analogs, oxidized form. For experimental details see Mordente et al.[14]

to delocalize redox-active low molecular weight (LMW) iron from otherwise inactive cellular stores.[24] Once delocalized in a LMW form, iron promotes lipid peroxidation through reaction mechanism(s) that may not depend any longer on $O_2^{\bullet -}$ and H_2O_2. This is the case of ADP-Fe(II) complexes, which initiate liposomal and microsomal lipid peroxidation by means of mechanisms that may be insensitive to SOD and/or catalase.[24,25] ADP-iron dependent lipid peroxidation probably proceeds through the oxidation of Fe(II) with oxygen and the formation of perferryl species [Fe(II)O_2-Fe(III)$O_2^{\bullet -}$] or poorly characterized Fe(II)-O_2-Fe(III) complexes.[24] In either case, lipid peroxidation best occurs when appropriate Fe(II):Fe(III) ratios are formed, although the molecular basis for such a requirement has yet to be defined precisely.[24,35] $IDBH_2$ effectively decreases the oxidation of ADP-Fe(II) by oxygen, thus precluding the initiation of lipid peroxidation by reactive species, which require Fe(III).[14] The rate of Fe(II) oxidation would not appreciably be affected by IDB, showing that iron is maintained in the reduced form by the hydroquinone moiety. In microsomes, lipid hydroperoxides generated on ADP/iron-dependent initiation of peroxidative processes are known to react with cytochrome P450. This process accompanies the destruction of cytochrome P450 and liberates lipid alkoxyl radicals (LO$^{\bullet}$), which further propagate lipid peroxidation.[36,37] $IDBH_2$ is able to inhibit lipid peroxidation not only by affecting ADP/iron initiated processes, but also by scavenging propagating species generated upon cytochrome P450 involvement.[14] Experiments employing cumene hydroperoxide (CUOOH) in place of ADP-Fe(II) confirm that the hydroquinone moiety inhibits cytochrome P450-dependent lipid peroxidation by scavenging alkoxyl radicals formed upon cytochrome P450 cleavage of CUOOH.[14] Idebenone can therefore mitigate the toxicity associated with iron delocalization and oxidative deterioration of biological membranes.

10.3 PHARMACOLOGICAL PROPERTIES AND THERAPEUTIC POTENTIAL OF COENZYME Q ANALOGS

Coenzyme Q_{10}, due to its bioenergetic capacity and/or antioxidant activity, has been widely used in the prophylaxis and therapy of a wide variety of pathological states,[1–3,38] although it has remained uncertain whether its deficiency is the cause or the effect of the disease state itself. On the contrary,

the pharmacological properties and the therapeutic potential of short chain quinones remain actually unexplored since these synthetic quinones have not been employed in *in vivo* studies except for idebenone, upon which this section will be essentially based.

Idebenone is currently administered to ameliorate cognitive status in patients with clinical history of stroke, Alzheimer's disease, and multiinfarct dementia.[39–41] Idebenone has been reported to improve cerebral energy metabolism,[39,42] to decrease excitotoxic neuronal degeneration,[43] and to stimulate nerve growth factor synthesis.[39,44] Idebenone also appears to minimize platelet formation of thromboxane[45] as well as the toxicity of oxidized low density lipoprotein to endothelial cells.[46] In so doing, idebenone inhibits platelet aggregation and contributes to the maintenance of vascular wall integrity and functions. The mechanism by which IDB exerts its pharmacological effects remains to be precisely defined, although it seems to be mainly related to its antioxidant activity, which is already appreciable at $\cong 2\ \mu M$, that is well in the range of idebenone plasma levels attainable in patients after oral supplementation.[39]

The central nervous system (CNS), which is highly dependent on aerobic ATP production, is the tissue most sensitive to oxidative damage, for at least four different reasons.[47] First, CNS accounts for less than 2% of body weight and yet it consumes up to 20% of total body oxygen, producing more superoxide radicals than other tissues. Second, neuronal membranes are very rich in easily peroxidable polyunsaturated fatty acids, and their antioxidant status is in some way lower than in other tissues. Third, the brain is extremely rich in iron and particularly prone to the deleterious consequences of LMW iron delocalization. As a matter of fact, the content of transferrin in cerebrospinal fluid is very low; hence it cannot sequester LMW iron and prevent iron-catalyzed oxidative damage. Finally, neurons accumulate epinephrine, norepinephrine, 3,4-dihydroxyphenylalanine, 6-hydroxydopamine, and melanins, all capable of reducing iron, which in turn reduces oxygen to $O_2^{\bullet -}$ and H_2O_2. Considering that CNS has very little superoxide dismutase, catalase, and glutathione peroxidase activities as compared to other tissue,[47] its antioxidant defenses are mainly based on nonprotein antioxidants. As a consequence, it can be easily understood why free radicals, which are normally involved in brain physiology, may readily induce the oxidative stress associated with almost all brain disorders, as evidenced by biochemical indexes of lipid peroxidation.[48] As discussed above, $O_2^{\bullet -}$ and H_2O_2 may trigger lipid peroxidation by generating more damaging species such as $ONOO^-$ (Figure 10.3), which is known to mediate neurotoxicity by excitatory amino acids such as glutamate.[23,49] Superoxide is also known to release iron from ferritin[50] or enzymes endowed with Fe-S clusters.[51] On the other hand, H_2O_2-dependent activation of hemoproteins may very likely occur when vascular damage and bleeding set the stage for a leakage of these proteins within tissues, as is in the case of brain trauma, stroke, or ischemia-reperfusion.[52] Furthermore, prolonged interactions of H_2O_2 with hemoproteins result in the irreversible denaturation of the heme pocket, breaking of the porphyrin ring, and consequent release of the iron coordinated therein.[52] Finally, H_2O_2 can activate myeloperoxidase (MPO) to compound I and II intermediates that oxidize tyrosine to tyrosyl radicals,[21,28] In terms of neuropathology, tyrosyl radicals might be formed and play some noxious role under conditions of ischemia-reperfusion and H_2O_2 formation by activated neutrophyls, macrophages, and glial cells.

The involvement of free radicals in many brain disorders opens the possibility for prevention or therapy by the targeted use of antioxidants.[53,54] Drugs or natural compounds are very often studied *in vitro* to evaluate whether they can be used as antioxidants *in vivo*. Two major criteria should be kept in mind when performing this type of determination. First, test compounds should scavenge free radicals at concentrations attainable in tissues and biological fluids. Second, the free radical-scavenging efficiency of a given compound should withstand comparison with other established antioxidants. Antioxidant interventions with vitamin E or CoQ_{10} may be limited by pharmacokinetic constraints. Coenzyme Q_{10} approaches high plasma levels but brain (or heart) uptake is rather poor.[55,56] It follows that CoQ_{10} supplementation is a good strategy to ameliorate oxidant damage within the plasma milieu (e.g., LDL oxidation) but not in the nervous (or cardiac) tissue. Likewise, vitamin E penetrates CNS slowly and may therefore fail to afford protection in neural

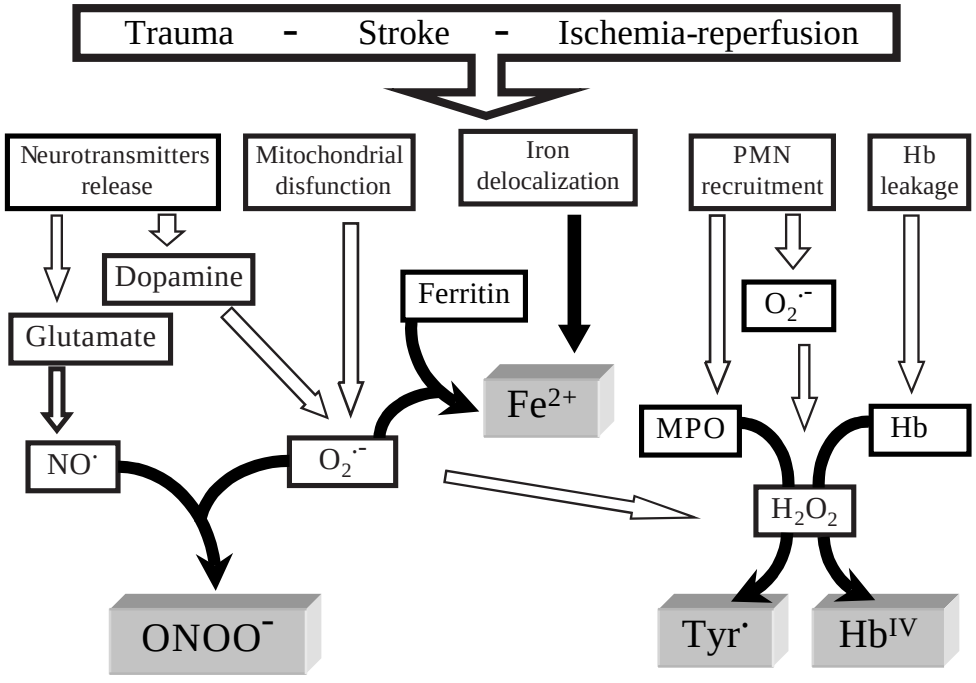


FIGURE 10.3 Oxidative stress and CNS damage: production of oxidant species, putative mediators of neurotoxicity.

cells acutely exposed to oxidative injury.[57] The clinical usefulness of vitamin E might therefore be confined to the long-term supplementation of patients affected by chronic degenerative processes, such as Alzheimer's disease.[58] These problems are not observed with idebenone, which distributes throughout the body and readily approaches similar concentrations in tissues and fluids.[39,59,60] The pharmacokinetic properties, as well as the antioxidant functions described in the preceding section, may help clarify the cytoprotective action of idebenone and its greater efficiency in comparison with vitamin E or CoQ_{10}.[61,62] Furthermore, since the antioxidant effect of quinones pertains to the hydroquinone form, the antioxidant efficiency of each compound is strictly dependent on its own rate of reduction. In this respect, the site(s) and mechanism(s) of IDB reduction are not yet defined. IDB might be reduced by the same mitochondrial dehydrogenases that reduce CoQ_{10}, although with the differences discussed in section 10.1, but also by other NADH:quinone reductases, recently isolated from membranes of intracellular organelles,[63] plasma membrane,[64] and cytosol,[65] which may reduce short chain coenzyme Q analogs much faster than CoQ_{10}. In this respect, idebenone might be viewed as a suitable drug for antioxidant interventions in free radical-mediated neurological diseases, being capable of intercepting free radicals both in aqueous phases and lipid-water interfaces. Finally, supporting the view of a wider biomedical application, idebenone has been successfully employed to protect organ transplants against oxidative damage, increasing the efficacy of organ preservation, maintaining donor organ quality, and preventing reperfusion injury.[62,66]

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Section 1D

Prooxidant Mechanisms

11 Coenzyme Q as a Generator of Reactive Oxygen Metabolites

Hans Nohl, Lars Gille, Andrey V. Kozlov, and Katrin Staniek

CONTENTS

11.1 INTRODUCTION

The physico-chemical properties of coenzyme Q are optimal for its functional involvement in energization of biomembranes. Conditions required for this function are the free diffusion within the various sections of a phospholipid bilayer in combination with the acceptance and release of single electrons and single protons. The intermediate reduction state of coenzyme Q between its fully oxidized and its fully reduced form is the semiquinone that can be obtained both from one-electron reduction of coenzyme Q (ubiquinone) and one-electron oxidation of the divalently reduced coenzyme Q (ubiquinol).

Among the three redox states of coenzyme Q, the semiquinone is by far the most reactive one. In biomembranes, where coenzyme Q undergoes redox-cycling, semiquinones formed are functionally in contact with the physiological reaction partners excluding undesired side effects. In mitochondria where the bioenergetic role of coenzyme Q was first recognized, four site-directed semiquinone pools were described communicating through the Q-pool by scalar electron transfer and transmembraneous proton conduction.[1–4] Binding to the respective redox partners warrants an orderly sequence of redox change and prevents an uncontrolled leakage of reducing equivalents. The matrix-directed proton gradient to which redox-active ubiquinones contribute is used to drive ATP synthesis. From this important bioactive function, which is associated with electron-cycling through the "Q-cycle," it is clear that protonation and deprotonation following electron acceptance and release is a major feature of coenzyme Q in biomembranes.[5] This bioactivity requires the physical stabilization of semiquinones such that redox change will exclusively occur with the respective physiological redox partner. The intensity of this interaction can be inferred from spin lattice

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relaxation behavior becoming visible through power saturation experiments in the ESR spectrometer. In this article it will be shown that undesired pathways of the transfer of reducing equivalents from semiquinones occur when stabilization of this radical is impaired or is not provided, as in membranes that do not recycle oxidized ubiquinones.

11.2 RESULTS AND DISCUSSION

11.2.1 Chemical Conditions Required for One-Electron Transfer from Coenzyme Q to Oxygen

The electronic configuration of the dioxygen molecule having two single electrons in the antibonding $2\pi^*$ orbitals forbids the transfer of a pair of electrons. Consequently, reductants of dioxygen must be one-electron donors and the direct reduction product is the superoxide radical. Ubiquinone species (UQ), which come into question as potential reductants, are semi- and divalently reduced ubiquinones.[6–8] Since these reduced UQ species can exist both in the protonated and in the deprotonated form, five different UQ species have to be considered as potential one-electron donors for dioxygen (Figure 11.1).

To prove which of the reduced UQ forms react with oxygen, it was necessary to establish conditions where oxidation of each of the potential reductants can be followed separately.

In contrast to oxidized UQ, which, on thermodynamic grounds, remains chemically unchanged in solution, UQ in any reduced state equilibrates with all possible redox states of UQ; e.g., ubisemiquinones undergo disproportionation, which leads to the simultaneous presence of oxidized and divalently reduced ubiquinones. Traces of oxidized UQ, which are always present in ubiquinol solutions, will trigger ubisemiquinone formation via comproportionation (Equation 11.1).

$$\text{Ubiquinone} + \text{Ubiquinol} \underset{\text{disproportionation}}{\overset{\text{comproportionation}}{\rightleftharpoons}} 2\ \text{Ubisemiquinones} \qquad (11.1)$$

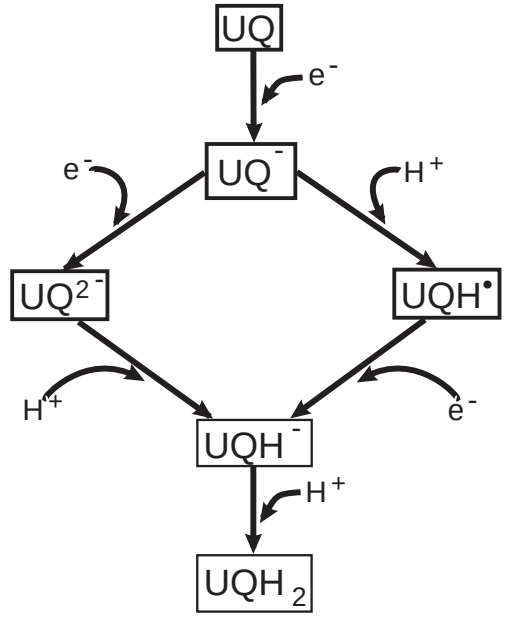


FIGURE 11.1 Stepwise reduction of ubiquinone yields five reduction forms with different polarities.

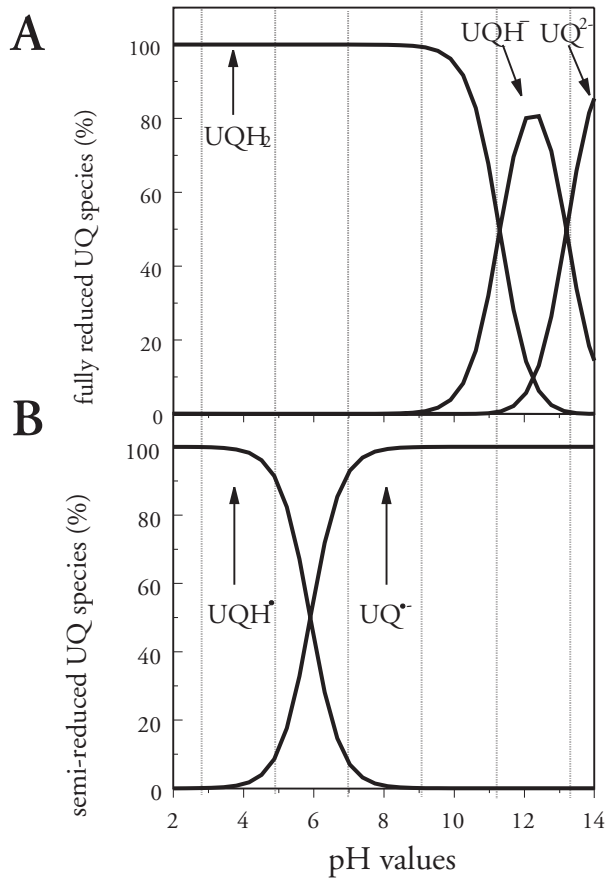


FIGURE 11.2 Computer simulations of thermodynamic equilibrium between protonated and deprotonated forms of fully reduced (A) and semireduced (B) UQ species at different pH values. Simulations were based on pK values ($pK(UQ^{\bullet -}/UQH^{\bullet}) = 5.9$; pK ($UQH^{-}/UQH_2$) = 11.3, ($pK(UQ^{2-}/UQH^{-}) = 13.2$), assuming that 100% of semireduced (B) or fully reduced (A) UQ species are present.

The widely differing pK values of semiquinones ($UQ^{\bullet -}/UQH^{\bullet} = 5.9$) and divalently reduced ubiquinones (UQ^{2-}/UQH^{-}) = 13.2) are other determinants that govern the presence of the respective reduced UQ species (Figure 11.2).[7,8] Based on these chemical interrelations, an experimental model system was developed allowing separate study of the interaction of the five reduced UQ species with oxygen. The existence and disappearance of the respective reduced UQ forms in the absence and presence of oxygen was made safe by photometric analysis in the case of divalently reduced ubiquinones and photometric plus ESR analysis in the case of ubisemiquinones (Figure 11.3). Reaction systems applied to identify ubiquinone species possibly interacting with dioxygen were carried out in a mixture of ethanol to solubilize UQ_{10} and 2.5% water for pH adjustment.

The possible interaction of any of the reduced UQ species with oxygen was concluded from oxygen-induced destabilization of the UQ species under study in combination with the accumulation of UQ the common oxidation product of all potential one electron donors. This is valid also for divalently reduced UQs, which decompose to ubiquinone following disproportionation of the first oxidation product ubisemiquinone.

Divalently reduced ubiquinones started to consume oxygen in the extreme alkaline pH range only. Oxygen removal from the reaction system was associated with accumulation of oxidized UQ. According to Figure 11.2, the predominant species at pH 12 is the semideprotonated ubiquinol,

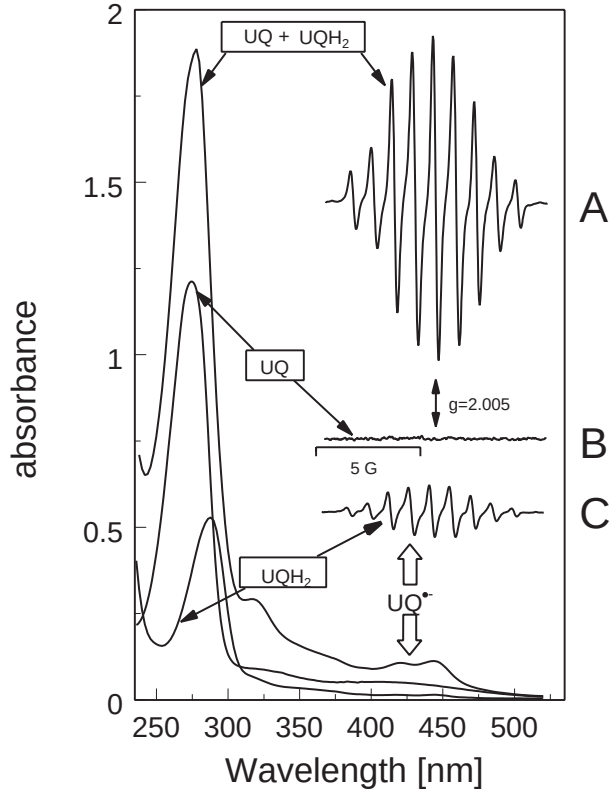


FIGURE 11.3 Optical and ESR spectra of ubiquinone 10 in the absence of oxygen dissolved in ethanol. UQH_2: Ubiquinol; UQ: Ubiquinone; UQ + UQH_2: comproportionation mixture yielding ubisemiquinones (Inserts: ESR signal of ubisemiquinones (A) resulting from stimulated comproportionation, (B) no signal formed, (C) adventitious ubisemiquinones from spontaneous comproportionation. Ubiquinone and ubiquinol (0.3 mM 1:1; M/M; l = 0.5 cm) at pH = 12.

while in the pH range at pH 13.5, dianionic ubiquinols predominate. The inevitable presence of traces of oxidized UQ in the original ubiquinol solution gives rise to the existence of contaminating semiquinones from comproportionation according to Equation 11.1.

Ubisemiquinones in the alkaline pH range exist predominantly in the deprotonated form. Thus, besides divalently reduced ubiquinones (UQ^{2-}/UQH^-) we have to consider ubisemiquinone anions as being responsible for O_2 removal from the reaction medium.

Identification of the species that interact with oxygen was possible when the ubisemiquinone content was increased at the expense of divalently reduced ubiquinones. This was possible when oxidized UQ was added to the original ubiquinol solution, which stimulated comproportionation thereby shifting the equilibrium in favor of ubisemiquinones (see Equation 11.1). In the alkaline pH range, ubisemiquinones obtained from stimulation of comproportionation are exclusively in the deprotonated form. Under these conditions both a clear stimulation of oxygen consumption and increased accumulation of ubiquinone in the reaction system was observed. This clearly indicates that O_2 consumption and reoxidation of divalently reduced ubiquinones are due to the presence of ubisemiquinone anions derived from comproportionation reactions, which are inevitable in the presence of traces of oxidized ubiquinone.

Figure 11.4 shows that in a nonaqueous medium, autoxidation does not occur unless water is present.[9] Contribution to the decrease of $UQ^{\bullet-}$ species by disproportionation was neglectable since in the absence of oxygen, $UQ^{\bullet-}$-related ESR signal remained nearly unchanged. Increasing the

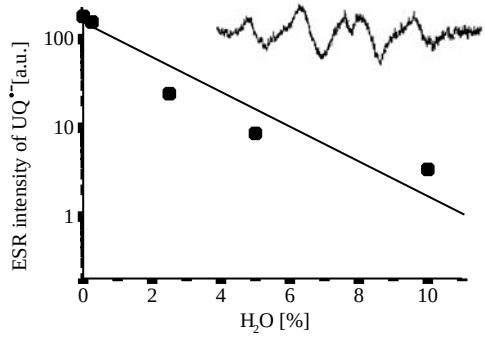


FIGURE 11.4 Increasing water content in acetonitrile dissolved ubisemiquinones stimulates autoxidation. Autoxidation was inferred from ESR signal decrease of $UQ^{\bullet -}$ and characteristic DMPO/•OOH adduct formation (insert). The concentration of UQ_0 in acetonitrile was 20 mM. Radical formation was initiated by addition of KO_2 (10 mM) and crown ether (10 mM). ESR settings were: sweep width 40 G, modulation amplitude 0.2 G, receiver gain $5 \bullet 10^3$, microwave power 2 mW, time constant 0.65 s, scan rate 14.3 G/min.

amount of water in the aprotic reaction system stimulates autoxidation of $UQ^{\bullet -}$. The inset shows the ESR spectrum of the DMPO/•OOH adduct obtained when autoxidation of $UQ^{\bullet -}$ occurred. The identity of the signal was made safe by control experiments in the same reaction buffer where KO_2 was used as a pure chemical $UQ^{\bullet -}$source. Since UQ molecules are exclusively present in the lipid phase of biomembranes, one can expect that the natural surrounding protects ubisemiquinones from autoxidation, although the pK value keeps them in the deprotonated state.

11.2.2 Interaction of Mitochondrial Ubiquinones with Molecular Oxygen ($O_2^{\bullet -}$ Radical Source)

Because the pK value for ubisemiquinones is below the physiological pH, ubisemiquinones participating in mitochondrial electron transfer do predominantly exist in the autoxidizable deprotonated form. Stabilization of ubisemiquinones involved in redox-cycling of the mitochondrial respiratory chain is a presupposition for an efficient energy gain from substrate oxidation. Redox-cycling ubisemiquinones operating in the respiratory chain of intact mitochondria form stable redox couples with their physiological oxidants. Steady state formation of ubisemiquinones in the respiratory chain requires a permanent flow of electrons to cytochrome oxidase where dioxygen is reduced to water. In our experiments oxygen was substituted with ferricyanide, which overtakes electrons from cytochrome *c* thereby allowing steady state formation of redox-cycling ubisemiquinones as in oxygen respiring mitochondria.[9,10] This experimental concept permits direct study of the effect of oxygen on electron-carrying ubisemiquinones. Signal heights of ubisemiquinone-related ESR spectra were identical regardless of whether O_2 or ferricyanide were used to keep the Q-cycle running (Figure 11.5a,b). Addition of oxygen to mitochondria respiring with ferricyanide as terminal electron acceptor did not affect the $UQ^{\bullet -}$ derived ESR signal (Figure 11.5c). The lack of any response to the absence or presence of oxygen reveals that autoxidation of ubisemiquinones is normally not to be expected in mitochondria.

We have recently developed a sensitive, noninvasive method for the detection of $O_2^{\bullet -}$ possibly released from mitochondria.[11] In agreement with the insensitivity of mitochondrial ubisemiquinones to oxygen, the results obtained from intact mitochondria were negative (Figure 11.6). $O_2^{\bullet -}$ radical-derived H_2O_2 was not found under any metabolic condition unless antimycin A (AA) was added, which inhibits the regular electron transfer through the Q-cycle. As shown before, autoxidation of ubisemiquinones requires the presence of deprotonated ubisemiquinones and the redox-cycling head group must be in contact with the aqueous phase. Ubisemiquinones

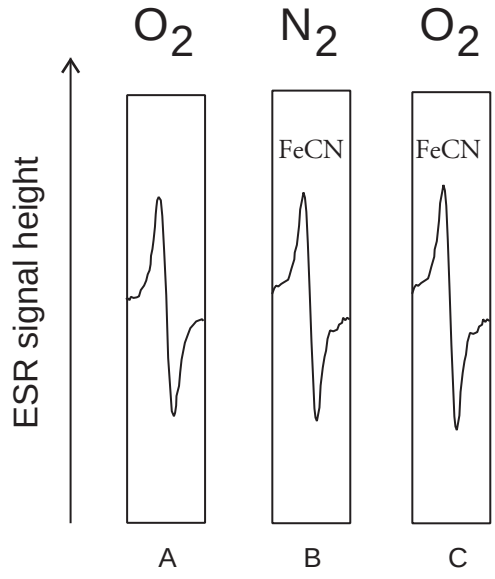


FIGURE 11.5 The effect of ferricyanide on mitochondrial ubisemiquinone population. ESR signal of $UQ^{\bullet -}$ in succinate-respiring mitochondria (A) under aerobic conditions, (B) oxygen was replaced by ferricyanide as mitochondrial electron acceptor and mitochondria were kept under nitrogen, (C) under aerobic conditions but in the presence of ferricyanide. The incubation mixture contained 3.2 mg/ml RLM, 24 mM fumarate/succinate 1:5, and 7.3 mM phosphate, and 12 mM $Fe(CN)_6^{3-}$ if required. Immediately after the addition of ferricyanide and succinate/fumarate (20/4 mM), the reaction mixture, which was placed in a quartz tube was frozen by liquid nitrogen and subjected to ESR measurements. ESR settings: microwave power 1 mW; frequency 9.46 GHz; modulation frequency 100 KHz; modulation amplitude 5G; receiver gain $2 \bullet 10^4$.

involved in mitochondrial electron transfer of the respiratory chain are estimated to be 6 to 10 Å distant from the surface of the lipid bilayer.[12] Since semiquinones are paramagnetic compounds, their distance from the cytosolic aqueous phase can be estimated from spin exchange experiments with an external paramagnetic spin probe. The paramagnetic center of ubisemiquinones operating within reach of the aqueous phase is expected to interact with external spin probes, thereby enhancing spin relaxation visible by power saturation alterations. Suitable spin probes must have high spin densities and should not chemically react with any electron carrier nor should they penetrate into the lipid core of the membrane. Considering these conditions, we selected potassium-chrome-oxalate (Cr^{3+}) for the localization of mitochondrial $UQ^{\bullet -}$ and gadolinium salt for the determination of the $UQ^{\bullet -}$ position in nonrespiring bilayers. Redox-cycling ubisemiquinones of intact mitochondria earlier shown to be insensitive to oxygen were also not affected by the external spin probe Cr^{3+}. In contrast, mitochondria subjected to conditions that establish the release of $O_2^{\bullet -}$ radicals from the respiratory chain were found to interact with the external paramagnetic spin probe (Figure 11.7). The coexistence of physical interaction of the paramagnetic center of mitochondrial ubisemiquinones with the electron spin of the paramagnetic probe in the cytosol and the chemical interaction with dioxygen reveals the involvement of the bordering water phase in the autoxidation reaction of $UQ^{\bullet -}$. As demonstrated, autoxidation requires ubisemiquinones in the deprotonated form. Water may favor the transformation of protonated to deprotonated ubisemiquinones by the addition of protons to H_2O (function of a base) and stabilization of the ubisemiquinone anion radical through solvation (cation function). This function would be in favor of autoxidation and prevent disproportionation, which requires protonated ubisemiquinones. In addition protonation of

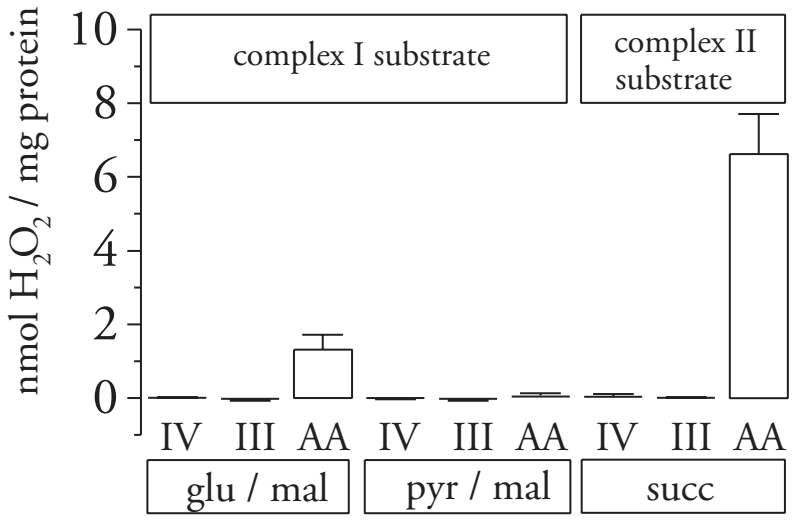


FIGURE 11.6 Determination of $O_2^{\cdot -}$ derived H_2O_2 generation in RHM respiring under various metabolic conditions, e.g., state IV (substrate only), state III (substrate plus ADP) or antimycin A-inhibited (AA) respiration. Experimental conditions: 0.3 M sucrose, 20 mM triethanolamine, 1 mM DETAPAC, 0.5 mg BSA/ml, pH 7.4 (incubation buffer), 4 mM P_i, 10 mM succinate (or 5 mM glutamate/5 mM malate or 2.5 mM pyruvate/2.5 mM malate, respectively), 2 μg/ml antimycin A (AA), 0.5 mg/ml mitochondrial protein. RHM were made to produce H_2O_2 in the absence of the detection system for 3 min at 25°C and separated by centrifugation (5 min; 9,000 g). The supernatant was placed into a fluorescence cell, 5 μM scopoletin were added and HRP-catalyzed (10 U/ml) fluorescence decrease (excitation: 366 nm, emission: 460 nm) was monitored. Data represent means ±SE of 3–9 independent mitochondrial preparations.

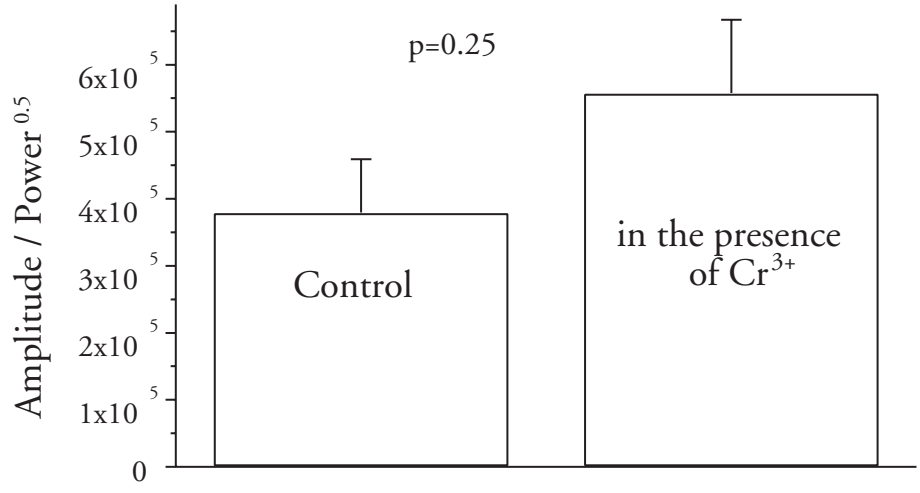
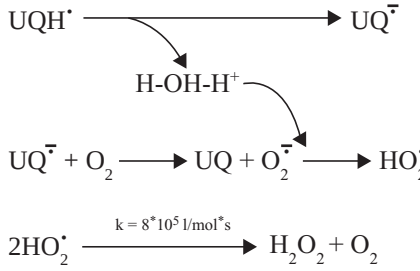


FIGURE 11. 7 Power saturation response of the ubisemiquinone ESR signal in succinate/fumarate-respiring RHM after preincubation with exogenous NADH in the absence and presence of Cr^{3+}. Final concentrations: RHM 16.85 mg protein/ml, NADH 10 mM (preincubation; 25 min at 4°C), succinate 20 mM, fumarate 4 mM, Cr^{3+} 50 mM. ESR measurements were carried out at 200 K using a flow dewar and a temperature control unit. The spectrometer settings were: microwave frequency 9.43 GHz, power 20 mW, modulation frequency 100 kHz, modulation amplitude 4 G, receiver gain $4 \cdot 10^5$, points 1024, time constant 0.655 s, scans 4, sweep 80 G, center field 3360 G, scan rate 57.2 G/min.

superoxide anions emerging from $UQ^{\bullet -}$ autoxidation stimulates the spontaneous dismutation that drives the reaction via H_2O_2.



(11.2)

(11.3)

(11.4)

Structural or functional alterations of mitochondria were found to establish conditions under which redox-cycling ubisemiquinones operate in contact with the cytosolic aqueous phase.

i) Derangement of the physical order of the phospholipid membrane structure was found to correlate quantitatively with the susceptibility of redox-cycling ubisemiquinones to oxygen (Figure 11.8). Since $UQ^{\bullet -}$ in membranes undergoing these alterations exhibited spin-spin exchange with hydrophilic spin probes, it may be assumed that water penetrates into the deranged lipid bilayer forming hydrophilic niches where $UQ^{\bullet -}$ radicals can readily undergo autoxidation. An example of the far-reaching impact this has on a regular electron transfer through the Q-cycle is the incorporation of various lipophilic xenobiotics into the lipid phase of the inner mitochondrial membrane (Figure 11.9).

ii) Totally different conditions without any physical alterations of the mitochondrial membrane led to $UQ^{\bullet -}$ autoxidation in mitochondria exposed to ischemia/reperfusion. Heart mitochondria were earlier shown to run a non-energy-linked pathway of electron transfer along all sections of the respiratory chain.[13] This redox chain is activated when cytosolic NADH levels are elevated over physiological values. Such a situation can be expected as a result of anaerobic glycolysis during anoxia or ischemia. NADH, which increases five-to sixfold in that case, is readily oxidized via the exogenous NADH dehydrogenase that catalyses the entrance of reducing equivalents into complex I.[14] The electron transfer from complex I involves a particular ubisemiquinone species that exhibits ESR characteristics different from the mitochondrial $UQ^{\bullet -}$ species reported earlier.[15] Power saturation experiments revealed a weak physical interaction (spin coupling) with complex I. This was concluded from the relatively low spin lattice relaxation requiring low microwave power absorption to achieve saturation conditions. Spin-spin interaction with the hydrophilic Cr_r^{3+} salt outside the lipid bilayer indicates that this novel $UQ^{\bullet -}$ species operates close to the cytosolic water phase. According to the above, conditions required for $UQ^{\bullet -}$ autoxidation activation of this particular $UQ^{\bullet -}$ species as a result of ischemia followed by reperfusion led to the release of $O_2^{\bullet -}$ radicals. Apart from this novel semiquinone species, which is assumed to exert a pathogenic role as an oxygen radical source in reperfusion injury of the heart, electron transfer from ubiquinol to the bc_1 complex is likely to be another site of single electron diversion from the respiratory chain to oxygen. This redox pair is the most vulnerable of all redox couples of the respiratory chain. Two single electrons from ubiquinol are transferred; one is recycling via the *b*-cytochromes into the Q-cycle while the second electron goes linearly to cytochrome oxidase via the Rieske-iron sulfur protein (Figure 11.10). Electron transfer at this site is associated with two deprotonation steps. Considering the standard free energy changes involved, it seems that deprotonation represents an activation barrier that must be overcome by kinetic activities associated with conformational changes of the two electron acceptor sites. We have some experimental evidence that an impediment of conformational adaptation to overcome kinetic restrains of electron bifurcation is a further prerequisite for an electron leakage from $UQ^{\bullet -}$ to oxygen out of sequence.[16]

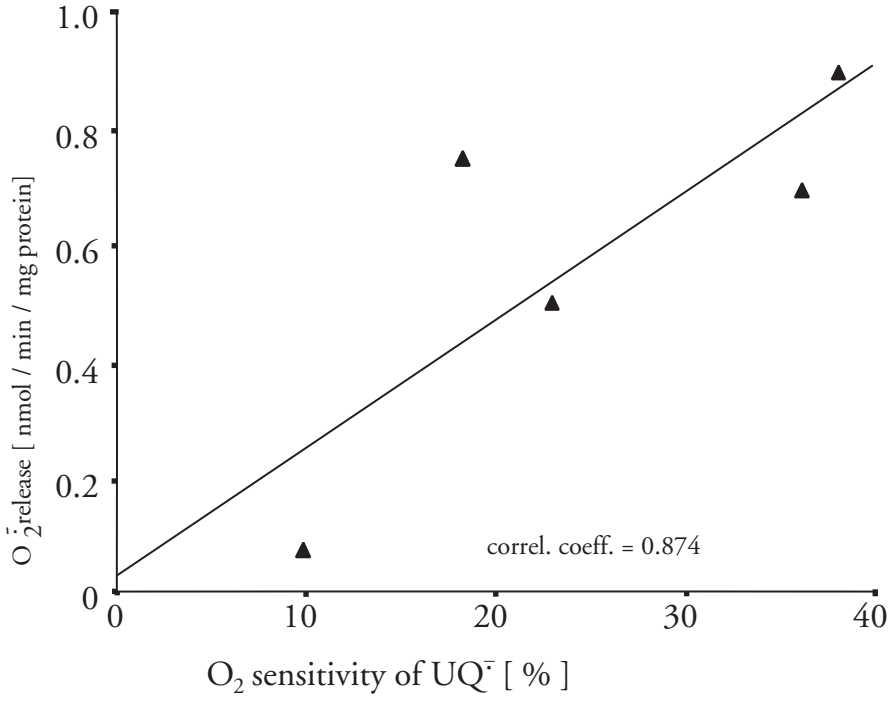


FIGURE 11.8 Correlation between oxygen sensitivity of redox-cycling ubisemiquinones and superoxide radical release from respiring mitochondria. O_2 sensitivity was elevated by increasing the fluidity of the lipid membrane through incorporation of increasing amounts of toluene. The fluidity change was assessed from spin labeling experiments with 5-doxyl-stearic acid incorporated in the inner mitochondrial membrane and calculation of the order parameters as in [15]. 3 mg of RLM were suspended in 3 ml of buffer. 100μl of toluene were added and the incubation system was gently stirred under air oxygen. After 4 min, the incubation procedure was stopped by diluting and after sedimentation, mitochondria were used for ESR experiments. The reaction medium contained 21.7 mg/ml RLM, 26 mM fumarate succinate 1:5, and 8 mM Pi. The O_2 sensitivity was calculated from ESR spectra in presence (O_2) and absence (N_2 + 13 mM $Fe(CN)_6^{3-}$) of oxygen. The spectrometer settings were: Scan range 100 G, modulation amplitude 5 G, receiver gain 1×10^5, microwave power 10 mW, time constant 0.65 s, scan rate 36 G/min, scans 15. $O_2^{\cdot-}$ formation rates were calculated from the SOD-sensitive cooxidation of epinephrine to adrenochrome measured photometrically at 480/575 nm. Other concentrations were identical with ESR experiments.

11.2.3 Interaction of Redox-Cycling Ubisemiquinones with Hydrogen-Peroxide ($HO^{\cdot}$ Radical Source)

Superoxide radicals, once released from the respiratory chain, form H_2O_2 both by spontaneous dismutation and more efficiently by SOD-catalyzed degradation to H_2O_2 and oxygen. We have shown earlier that 80% of total $O_2^{\cdot-}$ generated are released in the form of H_2O_2 following SOD-catalyzed dismutation in the matrix space. The presence of catalase in the matrix in addition to SOD does not prevent a permanent flux of H_2O_2 from the matrix into the extramitochondrial compartment.[17] Consequently, H_2O_2 diffuses across the inner mitochondrial membrane (diff. const. = 105 nm/min) allowing a collision with electron-transferring components of the respiratory chain. Using DMPO spin trapping technique, ESR spectra were obtained from succinate respiring mitochondria in the presence of antimycin A, which can be attributed essentially to the formation of DMPO/$^{\cdot}$OH radical adducts overlapped by DMPO/$^{\cdot}$OOH adducts (Figure 11.11). The hyperfine

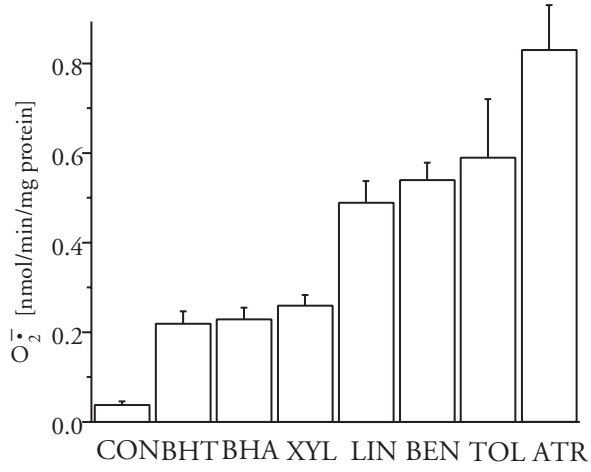


FIGURE 11.9 Incorporation of various xenobiotics into the inner mitochondrial membrane triggers superoxide radical release ($O_2^{\bullet-}$). Superoxide generation rates were inferred from superoxide dismutase-sensitive cooxidation of epinephrine as in Figure 11.8. BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; XYL, xylenol; LIN, lindane; BEN, benzene; TOL, toluene; ATR, atrazine; CON, controls. The xenobiotic (100 μM final concentration) was added to the RHM solution (30 mg /ml protein) at 0°C and incubated for 20 min.

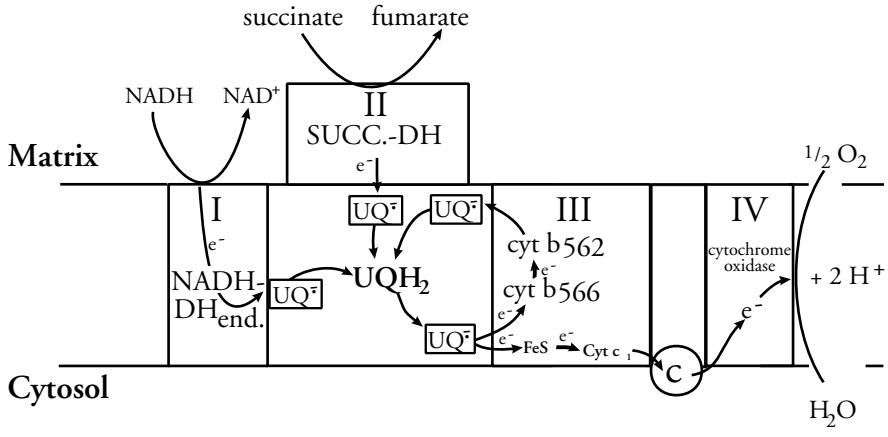


FIGURE 11.10 Scheme of ubiquinone redox-cycling in mitochondria. Ubiquinones shuttle electrons by two consecutive single electron transfer steps from complex I (NADH: ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase) to complex III (cytochrome *b*/cytochrome c_1 complex). Divalently reduced ubiquinone (UQH_2) has the function of a pool providing a pair of electrons for reduction of the bc_1 complex. Bifurcation of electron transfer supplies one electron to cytochrome b_{566} (low potential cytochrome *b*), which recycles the electron via cytochrome b_{562} (high potential cytochrome *b*) to ubiquinone while the second electron follows a scalar transfer to cytochrome oxidase of complex IV. The second UQH_2 contributes the second cycling electron to complete the reduction of $UQ^{\bullet-}$ to UQH_2 (Q-cycle).

splitting characteristic of the $HO_2^{\bullet}$ adduct disappeared when SOD was added and the intensity of the DMPO/•OH-related quartet signal decreased by around 20%. This indicates a small fraction of $O_2^{\bullet-}$ bound to DMPO giving rise to the apparent presence of HO• radicals by inner molecular derangement of the adduct, which results in the same ESR quartet as HO• directly added.

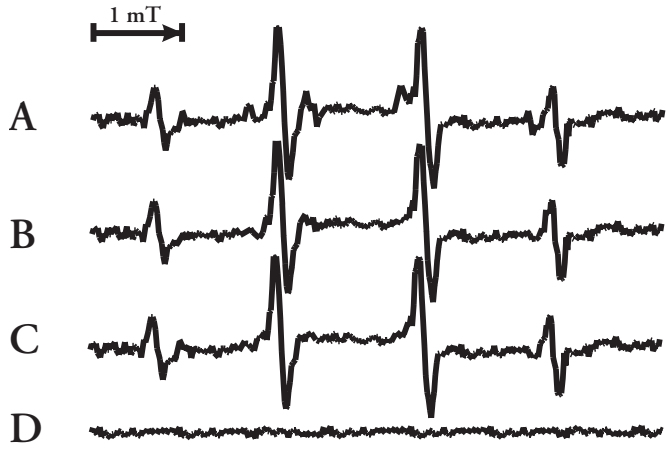


FIGURE 11.11 ESR spectra of the DMPO/•OH adduct formed by trapping of $HO^{\bullet}$ radicals from respiring mitochondria; no addition (A), + SOD (B), + catalase (C), myxothiazol (D). Mitochondria were uncoupled by freezing and subsequent thawing. Mitochondrial protein (1.5 mg) was suspended in 300 μl reaction medium [0.15 M KCl − 0.5 mM KH_2PO_4-buffer (pH 7.4)] in the presence of 6 mM succinate, 1 μg antimycin A and 0.3 M DMPO.

The ESR signal intensity of the HO•–related DMPO adduct was insensitive to catalase added from the outside. Since catalase has no access to H_2O_2 while permeating the mitochondrial membrane, HO• formation from H_2O_2 is likely to occur in contact with single electron carriers of the respiratory chain supplying electrons required for homolytic cleavage. Support for this concept also came from the insensity of HO• release to iron chelators normally preventing Fenton-type reactions and the total disappearance of the DMPO/•OH adduct when the ubiquinol bc1-redox couple was intercepted by myxothiazol.

The involvement of mitochondrial electron flow as an essential stage in the formation of HO• spin adducts was assayed by competitive inhibition of succinate oxidation on addition of malonate. HO• radical formation was measured with uncoupled rat heart mitochondria in the presence of antimycin A. Redox changes of cytochrome b_{566} the respiratory component before the antimycin A stop were followed at various malonate/succinate poises and compared with the ESR signal height of DMPO/•OH adduct formation. Figure 11.12 shows the inhibition of succinate-induced reduction of cytochrome b_{566} at increasing malonate concentrations. Inhibition of cytochrome *b* reduction following malonate-related inhibition of electron flow into the respiratory chain was reflected by the decrease of HO• radical formation. When the malonate/succinate ratio was 10, both the reduction state of cytochrome *b* and the intensity of DMPO/•OH adduct formation were inhibited by 80%.

The involvement of $UQ^{\bullet -}$ in reductive homolytic cleavage of H_2O_2 was based on the following experiments. Monovalent reduction of quinone (UQ_0) was performed chemically in acetonitrile using a superoxide-generating system (KO_2 in crown ether) as a single electron source. The existence of the respective semiquinone form was inferred from ESR spectroscopy (Figure 11.13A). In the absence of contaminating water and oxygen, the semiquinone radical remained stable for several hours. The formation of stable semiquinone complexes with contaminating transition metals could be discounted because of the presence of iron and copper chelating compounds. These experiments show that acetonitrile is an appropriate solvent, allowing monovalent electron transfer from superoxide radicals to quinones with the subsequent generation of stable semiquinone radicals.

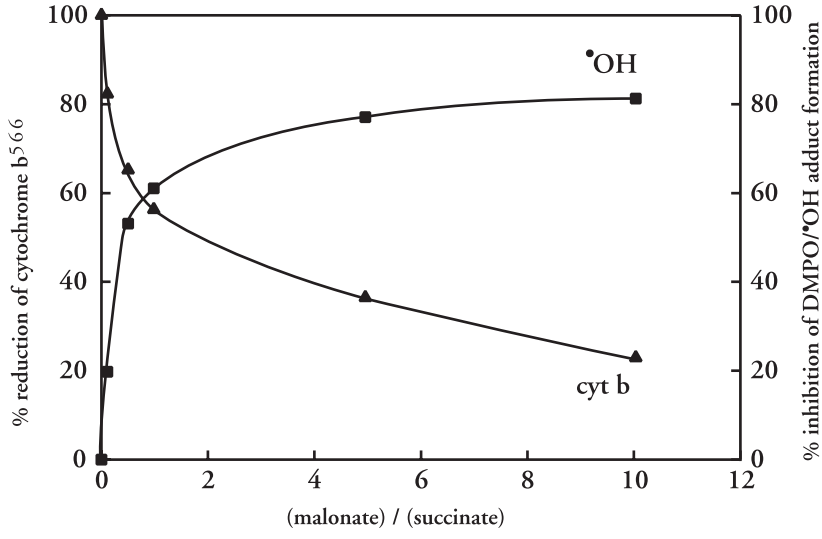


FIGURE 11.12 Involvement of the activity of electron transfer components in the formation of free HO• radicals. Succinate-induced respiration was down regulated by addition of increasing amounts of malonate (as inhibitor of succinate oxidation). Quantitative changes in DMPO/•OH adduct formation (■) was calculated from the intensities of the related ESR spectra. Values for redox changes of cytochrome b_{566} (▲) were taken from double beam spectra (Aminco DW-2 spectrophotometer) at 575–566 nm.

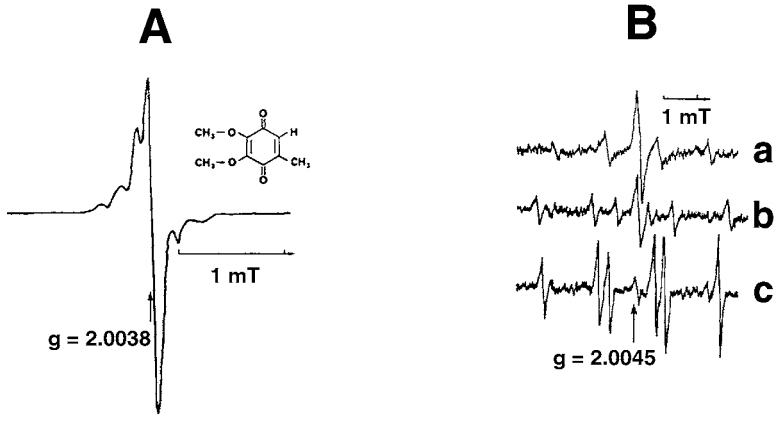


FIGURE 11.13 ESR spectra of ubisemiquinone radicals (A) and ESR signals of DMPO spin adducts (B) obtained by a reaction of ubisemiquinones with (a) 1.5 mmol/l H_2O_2, (b) in the presence of 1 mol/l ethanol, and (c) 1.5 mol/l formate. Generation of semiquinones from UQ was performed in acetonitrile in the presence of 10 mmol/l KO_2 dissolved in 20 mmol/l crown ether. Spin adduct formation of reaction products of semiquinones with H_2O_2, ethanol and formate was followed in a 125 mmol/l KCl, 50 mmol/l HEPES buffer, pH 7.4. The reaction was started upon mixing H_2O_2 with semiquinone in acetonitrile at room temperature. All reaction media were saturated with oxygen-free nitrogen and contained 6 mmol/l UQ_0; DMPO: 150 mmol/l, deferoxamine: 2 mmol/l; bathophenanthroline sulfonate: 1 mmol/l final concentrations. Microwave power: 12 mW (A, B); modulation amplitude: 0.1 mT (A); 0.04 mT (B).

Figure 11.13B shows ESR spectra resulting from (a) $HO^{\bullet}$, (b) ethyl-, and (c) carbonyl-radical spin adducts with the spin trap 5,5-dimethyl-pyrroline-N-oxide (DMPO). $HO^{\bullet}$ radicals became detectable when H_2O_2 was added to semiquinone radicals in the reaction medium (KCl-HEPES/acetonitrile; 70/30 v/v; pH 7.4). In the presence of ethanol, $HO^{\bullet}$ radicals were scavenged giving rise to the formation of ethyl radicals.[18] The presence of formate led to the formation of carbonyl radicals.

In the absence of semiquinones, hydroxyl radicals were not detected; also the absence of iron chelators did not result in the formation of detectable traces of $HO^{\bullet}$ radicals following the addition of H_2O_2. These studies strongly support the idea that mitochondrial generation of $HO^{\bullet}$ radicals is a consequence of $UQ^{\bullet-}$ autoxidation followed by $UQ^{\bullet-}$ catalyzed homolytic cleavage of $O_2^{\bullet-}$ derived H_2O_2. Although iron cannot be totally excluded as a catalyst for H_2O_2 degradation in mitochondria, the presence of redox-cycling semiquinones may be sufficient for the establishment of a cascade of oxygen activation steps ultimately causing $HO^{\bullet}$ radical formation.

$$2O_2 + 2UQ^{\bullet-} \longrightarrow 2O_2^{\bullet-} + 2UQ \qquad (11.5)$$

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (11.6)$$

$$H_2O_2 + UQ^{\bullet-} \longrightarrow HO^{\bullet} + HO^- + UQ \qquad (11.7)$$

Experimental support for the involvement of mitochondrial $UQ^{\bullet-}$ radicals in the reductive homolytic cleavage of H_2O_2 (according to Eq. 11.7) comes from the response of cytochrome b_{566} reduction to H_2O_2 when added to mitochondria (Figure11.14). Conclusions on an interaction of H_2O_2 with mitochondrial $UQ^{\bullet-}$ are based on the observation that in antimycin-inhibited respiration, redox potentials of b type cytochromes are governed by the ubiquinol/ubisemiquinone redox couple. Oxidants such as oxygen may increase antimycin-insensitive oxidation of ubisemiquinone to ubiquinone and consequently an extra-reduction of the b-type cytochromes will occur under these conditions. In order to prevent undesired effects of oxygen, reduction of cytochrome b_{566} was followed under anaerobic conditions using catalase-inhibited mitochondria. After reduction of cytochrome b_{566} with succinate and antimycin A, an additional increase in the reduction state of cytochrome b_{566} could be induced by adding H_2O_2 to the suspension. Stirring of the reaction medium also had a small effect on cytochrome *b* reduction, indicating contamination with atmospheric oxygen. The requirement of electron flow through the respiratory chain in combination with H_2O_2-inducible oxidation of redox-cycling $UQ^{\bullet-}$ reveals the catalytic function of the latter in the homolytic cleavage of H_2O_2 and subsequent $HO^{\bullet}$ formation.

We have recently observed that $UQ^{\bullet-}$ associated with lysosomal membranes undergo NADH-dependent redox-cycling in the presence of oxygen.[19,20] UQ is reduced by the subsequent transfer of two single electrons associated with a unilateral proton transport into the lysosomal lumen. Ubisemiquinones involved in this novel redox chain are likely to interact with dioxygen. It seems that in analogy to mitochondria, lysosomal $UQ^{\bullet-}$ may also exert a double role as a one-electron reductant of dioxygen and a one-electron reductant of H_2O_2 since $HO^{\bullet}$ radicals were detected by means of spin trapping with DMPO when UQ redox-cycling was initiated.

11.2.4 Antioxidant-Derived Ubiquinol Metabolites with Prooxidant Activities

When UQH_2 is present in peroxidizing lipid membranes, $UQ^{\bullet-}$ are formed from an interaction with lipid radicals or α-tocopherol radicals.[20] In contrast to $UQ^{\bullet-}$ operating as redox carriers of the respiratory chain, antioxidant-derived $UQ^{\bullet-}$ species in biomembranes, which have no recycling system for UQ, are rather unstable. Spin-spin interactions with external Gd^{3+} revealed that antioxidant-derived

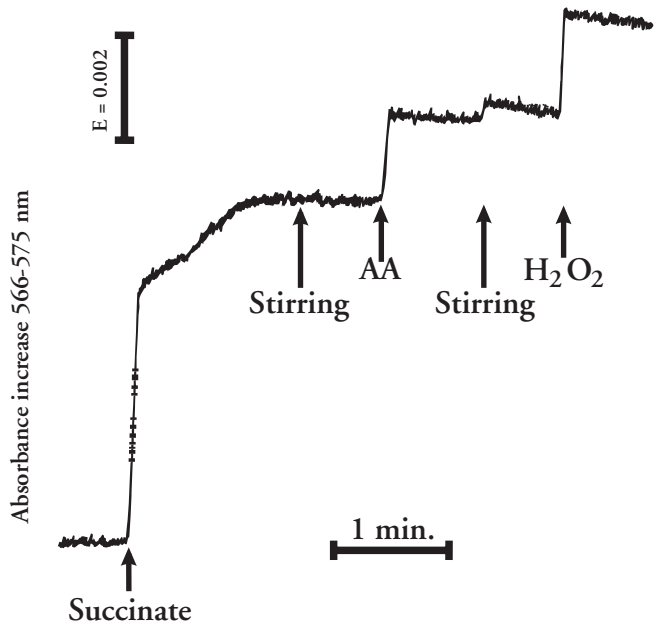


FIGURE 11.14 Influence of H_2O_2 on the reduction of cytochrome b_{566}. Experimental conditions: antimycin A-inhibited rat heart mitochondria (1.5 mg/ml) were suspended in a KCl/Hepes buffer, pH 7.4, and perfused with oxygen-free nitrogen. The anaerobic suspension was kept in a gastight cuvette and placed in a double beam spectrophotometer. Oxygen from added solutions was also removed by oxygen-free nitrogen, prior to their addition to the sample cuvette. The traces of the experiments are representative of 5 other measurements. Additions: succinate 6 mM, H_2O_2 0.15 mM final concentrations; antimycin A (AA) 1.5 μg.

$UQ^{\bullet -}$ were present in close proximity to the bordering water phase. Thus autoxidation was expected to be responsible for rapid destabilization of UQH_2-derived ubisemiquinones in peroxidizing membranes. The detection of H_2O_2 release from peroxidizing liposomes preloaded with UQH_2 confirmed this assumption (Figure 11.15). In analogy to mitochondrial $UQ^{\bullet -}$ antioxidant derived $UQ^{\bullet -}$ in liposomes were found to react both with inorganic and organic hydroperoxides giving rise to the formation of $HO^{\bullet}$ and alkoxyl radicals, respectively (Figure 11.16).[20] Indirect evidence for the implication of ubiquinol-derived prooxidants in the antioxidant activity of coenzyme Q comes from the following observations: i) in a homogenous reaction system, both UQH_2 and α-tocopherol remove linoleic hydroperoxy radicals with almost identical rate constants,[20] and ii) in contrast, α-tocopherol protects peroxidizing lysosomal membranes more efficiently than ubiquinol. The divergency between α-tocopherol and UQH_2 was more pronounced the more antioxidants were present, suggesting increasing prooxidant formation responsible for the declining protective effect of coenzyme Q.

11.2.5 Interaction of Redox-Cycling Ubisemiquinone with Nitrite (Nitrite Reductase Activity)

Nitrite (NO_2^-) is the major stable oxidation product of nitric monoxide (NO). Reduction to its bioactive form requires the establishment of a redox couple in which single electron transfer to NO_2^- is thermodynamically favored. Based on thermodynamics, all mitochondrial one-electron carriers on the reductant site of the Rieske-iron sulfur protein may be candidates for nitrite reduction when accessible (see Figure 11.10). Submitochondrial particles that are deprived from endogenous substrates for respiration, recycle nitrite to NO following initiation of respiration both with complex I and complex II substrates (exp. not shown). Endogenous substrates present in intact mitochondria keep the respiratory chain running sufficiently such that NO_2^- becomes totally reduced. The addition

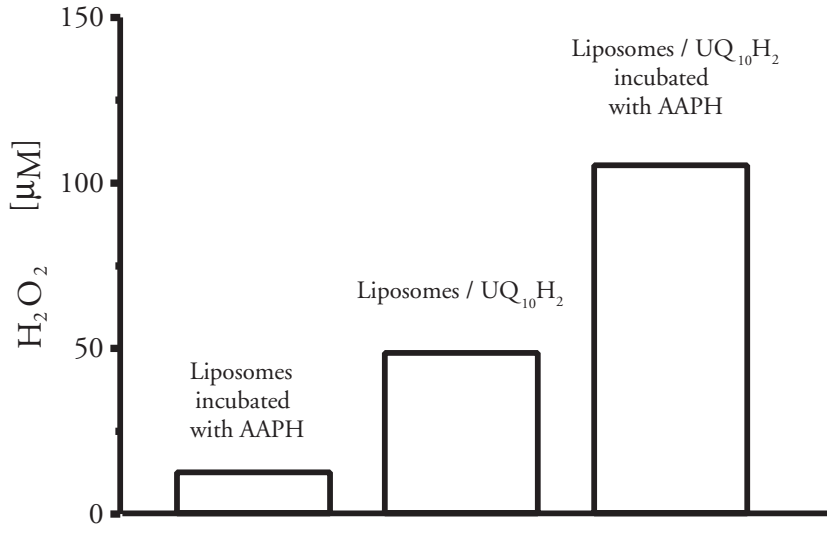


FIGURE 11.15 H_2O_2 formation as by-product of antioxidant activities of $UQ_{10}H_2$ in liposomes subjected to conditions of LPO. (A) Peroxidizing liposomes without $UQ_{10}H_2$, (B) liposomes containing $UQ_{10}H_2$, (C) liposomes containing $UQ_{10}H_2$ after AAPH-induced LPO. The amount of H_2O_2 was assessed after two hours of incubation (37°C) from the catalase-sensitive fluorescence decay of scopoletine (Excitation 350 nm and emission 460 nm) catalyzed by horseradish peroxidase (HRP). Concentrations: Liposomes 20 mg/ml (soybean phosphatidyl choline/$UQ_{10}H_2$ molar ratio 20:1), in air-saturated buffer (P_i 50 mM, pH 7.4), 1 mM DTPA, 10 mM AAPH. H_2O_2 assay: scopoletine 1.5 μM, HRP 1.25 μM.

of external substrates for respiration did not stimulate nitrite reduction. The existence of NO was inferred from nitrosylation of deoxyhemoglobin giving a characteristic triplet ESR signal (Figure 11.17). In contrast to mitochondrial $O_2^{\bullet -}$ generation, the presence or absence of antimycin A(AA) was not critical for nitrite reduction. Complete inhibition of nitrite recycling required an interference in the complex transfer of reducing equivalents from ubiquinol to the bc1-complex. This was possible with myxothiazol, which inhibits the bifurcation of electron transfer both to cytochrome oxidase and to the Q-cycle via low potential cytochrome *b*. Ubisemiquinones are essentially involved in this electron branching process.

11.3 CONCLUSIONS

Ubiquinones exert bioenergetic functions in coupled redox systems such as the respiratory chain of mitochondria and, as only recently demonstrated, in lysosomes.[19] This biological activity is related to biomembranes where protons are separately translocated across the axis of the membrane while electrons are transferred along the axis of the membrane to the respective redox partners. The latter stabilize an otherwise highly unstable semiquinone form, which is the essential intermediate in all redox-cycling activities of UQ. Changes in the polarity of reduced ubiquinones, which are present in the charged and uncharged form, drives the unidirectional proton transfer. Unilateral proton translocation contributes to the energy source required in mitochondria for ATP synthesis and to acidification in lysosomes to arrest biomolecules for enzymatic degradation. Alternative activities, which have increasingly focused the interest of many laboratories, are all related to reactive oxygen species. Prooxidant formation is not expected to occur in mitochondria under physiological conditions while biomembranes, which do not have recycling systems for UQ, may become a source of reactive oxygen species when ubiquinol exerts antioxidant functions. Ubisemiquinones are the most vulnerable intermediates of redox-cycling ubiquinones readily

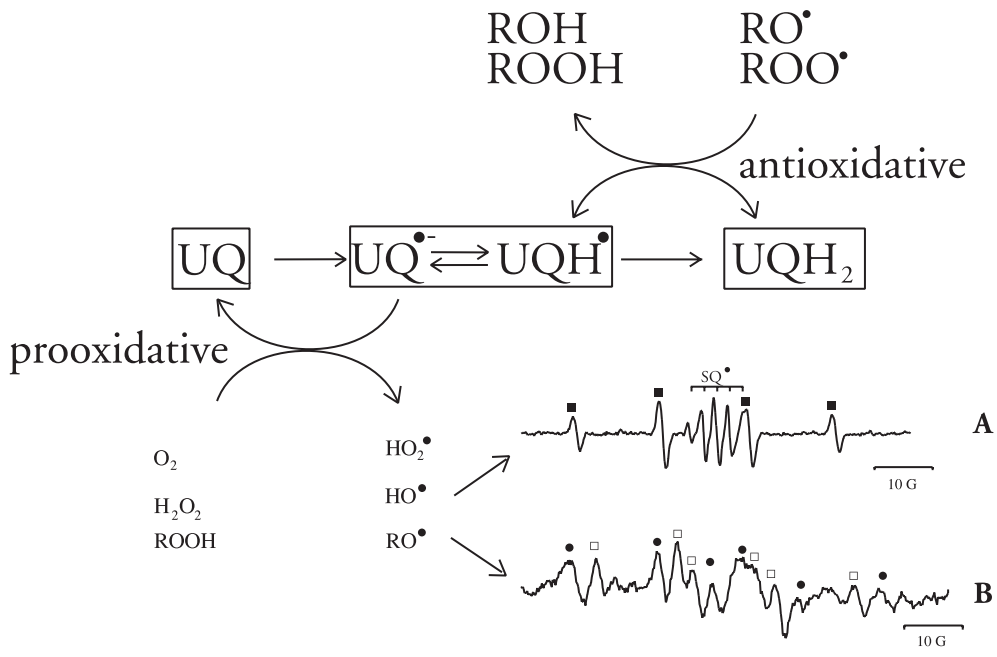


FIGURE 11.16 The compulsory development of prooxidant activities of ubisemiquinone $UQ^{\bullet -}$ emerging from the antioxidant function of UQH_2. ESR spectra of DMPO spin adducts observed during reaction of $UQ^{\bullet -}$ with H_2O_2 (Spectrum A), and linoleic acid hydroperoxide (ROOH) (Spectrum B). Based on computer simulations, ESR spectra indicate the presence of a DMPO/•OH adduct (■) superimposed by the $UQ_0^{\bullet -}$ signal. Spectrum b represents a combined DMPO/•CH_2R adduct (●) and DMPO/•C(O)R adduct (□). $UQ_0^{\bullet -}$ were obtained when xanthine (X) and xanthine oxidase (XOD) were mixed with 2,3-dimethoxy-5-methyl-1,4-benzoquinone (UQ_0; ubiquinone 0) in the absence of oxygen at 25°C. Concentrations: in argon-saturated buffer (P_i 50 mM, pH 7.4), 1 mM DTPA, X 5 mM, XOD 0.04 U/ml, UQ_0 0.1 mM, DMPO 9.3 mM, H_2O_2 1 mM, ROOH 1 mM. ESR settings: microwave power 20 mW, modulation amplitude 1 G, sweep 80 G, center field 3492 G, scan rate 229 G/min, time constant 0.02 s, receiver gain $5 \cdot 10^5$, microwave frequency 9.4–9.8 GHz, scans 1, modulation frequency 100 kHz.

undergoing autoxidation if not stabilized through binding to physiological redox partners or when operating in contact with the water phase. In mitochondria, deviation of electrons from $UQ^{\bullet -}$ to oxygen occurs following physical membrane alterations. Alterations must be such that $UQ^{\bullet -}$ become accessible from the aqueous phase and that the regular interactions of $UQ^{\bullet -}$ with the natural redox partners is affected. Prooxidant formation in mitochondria is therefore a pathophysiological side effect which also occurs when the nonenergy-linked electron transfer pathway is activated following NADH accumulation as a result of ischemia. The $UQ^{\bullet -}$ species involved in this case has particular properties favoring autoxidation. Regardless of the trigger transforming mitochondria to O_2-radical generators, compulsorily bioenergetic activities are reduced. The molecular basis is the decrease of the transmembraneous proton gradient by increasing proton conduction or proton dissipation as a consequence of cytosolic NADH oxidation.[15] In addition, nitrate reductase activity recently described by our group will ultimately affect the energy balance. NO formed in the metabolic compartment of mitochondria is likely to bind to cytochrome oxidase, which down-regulates energy-linked respiration, and as reported elsewhere, will also give rise to mitochondrial radical release. This may be of pathophysiological significance under conditions of nitrite accumulation, such as ischemia or the development of nitrate tolerance in patients treated with NO-donating drugs.

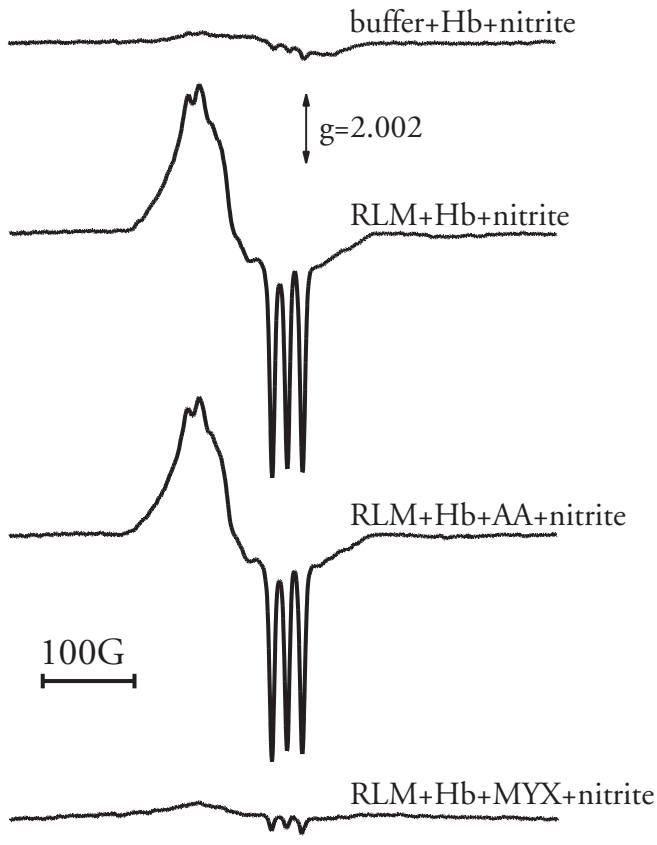


FIGURE 11.17 ESR spectra observed after 2 h incubation of rat liver mitochondria (RLM) or buffer (control) with nitrite in the presence of deoxyHb. The experimental medium contained: 5 mg of protein/ml RLM; 2.5 mM glutamate plus 2.5 mM malate, 250 μM Hb, 50 μM $NaNO_2$; 0.25 M sucrose, 20 mM triethanolamine, 1 mM EDTA, and 10 mM K_2HPO_4, pH = 7.25. ESR spectra were recorded at liquid nitrogen temperature with a Bruker EMX spectrometer under the following conditions: microwave frequency 9.43 GHz, modulation frequency 100 kHz, microwave power 20 mW, modulation amplitude 5 G; gain 10^5.

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Part 2

Nutritional Aspects of Coenzyme Q

Section 2A

Biosynthesis and Nutritional Sources

12 Genetic Analysis of Coenzyme Q Biosynthesis

Tanya Jonassen and Catherine F. Clarke

CONTENTS

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12.1 OVERVIEW OF COENZYME Q BIOSYNTHESIS

Coenzyme Q (ubiquinone or Q) is a prenylated benzoquinone lipid that functions in the electron transport chains of mitochondria in eukaryotes and plasma membranes in prokaryotes. Coenzyme Q is redox active and plays an important role as a lipid soluble antioxidant. In general, cells rely on *de novo* synthesis for their supply of Q,[1,2] and much progress has been made in the characterization of Q biosynthesis. It was work with Rhodospirillum rubrum, that allowed Karl Folkers and colleagues to identify the key Q-biosynthetic intermediates.[3] Elegant studies by Gibson and colleagues with Q-deficient mutant strains of *Escherichia coli* (containing mutations in the ubiA - ubiH genes)[4] enabled the steps of *E. coli* Q biosynthesis to be identified and ordered as shown in Figure 12.1. Biosynthesis begins with formation of a polyisoprenoid tail that contains a variable number of isoprene units (designated as *n*) depending on the species, and 4-hydroxybenzoic acid. After their covalent linkage to form the 3-polyprenyl-4-hydroxybenzoic acid intermediate (compound **5**, Figure 12.1), all subsequent steps involve the modification of the aromatic ring. The specific sequence of reactions differs in prokaryotes and eukaryotes. In *E. coli*, compound **5** undergoes decarboxylation, hydroxylation, and then methylation. Studies with Q-deficient mutants

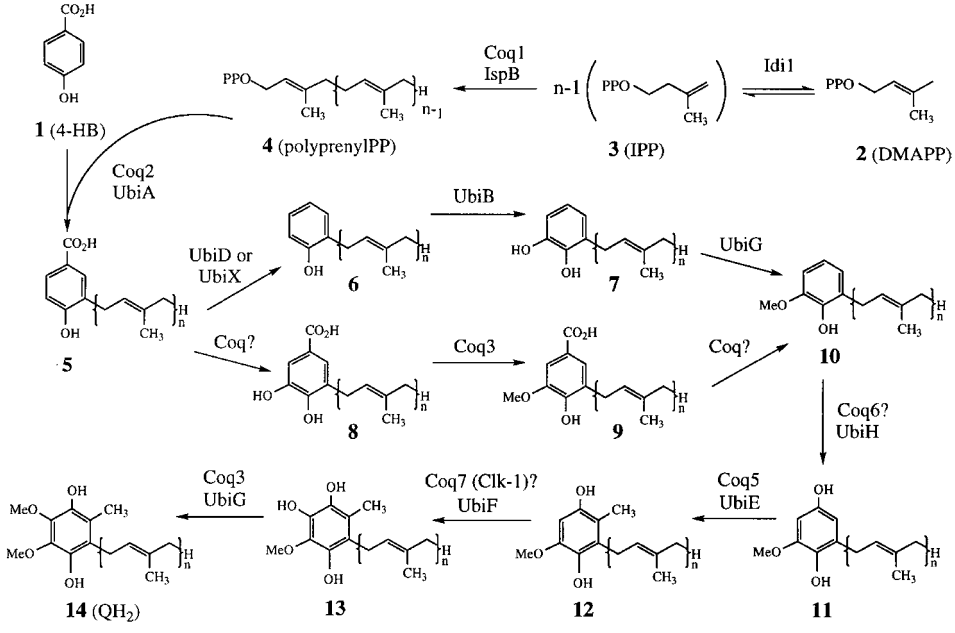

FIGURE 12.1 The proposed pathway of Q biosynthesis. Dimethylallyl diphosphate (**2**) and isopentenyl diphosphate (**3**) provide the precursors for the assembly of all-trans polyprenyl diphosphate (**4**), as catalyzed by Coq1 (*S. cerevisiae*) or IspB (*E. coli*). After formation of 3-polyprenyl-4-hydroxybenzoic acid (**5**), by the *p*-hydroxybenzoic acid:polyprenyltransferase (Coq2 or UbiA), the proposed biosynthetic pathways for Q in eukaryotes and in prokaryotes are thought to diverge. The other intermediates in the pathway are 2-polyprenylphenol (**6**); 2-polyprenyl-6-hydroxyphenol (**7**); 3,4-dihydroxy-5-polyprenylbenzoic acid (**8**); 3-methoxy-4-hydroxy-5-polyprenylbenzoic acid (**9**); 2-polyprenyl-6-methoxyphenol (**10**); 2-polyprenyl-6-methoxy-1, 4-benzoquinol (**11**); 2-polyprenyl-3-methyl-6-methoxy-1,4-benzoquinol or 5-demethoxyubiquinol (**12**); 2-polyprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol or demethyl-QH_2 (**13**); coenzyme Q_nH_2 (**14**). Compounds **11** and **13** are hypothetical intermediates in *S. cerevisiae* Q biosynthetic pathway, as is compound **7** in *E. coli*. In *S. cerevisiae*, $n = 6$ and compound **5** is referred to as 3-hexaprenyl-4-hydroxybenzoate (HHB). *E. coli* gene products are identified as Ubi (and also include IspB); *S. cerevisiae* gene products are identified as Coq (and also include Idi1). (From Clarke, C. F., *Protoplasma*, in press, 2000. With permission.)

of *Saccharomyces cerevisiae* indicate the order as hydroxylation, methylation, then decarboxylation.[5,6] After formation of compound **10**, the prokaryotic and eukaryotic pathways converge with the final hydroxylation and methylation steps leading to the fully substituted hydroquinone, Q.

The *E. coli ubi* mutant strains are unable to grow aerobically on nonfermentable carbon sources such as succinate or malate.[7] The *coq* mutant strains of *S. cerevisiae* harbor mutations in one of the *COQ* genes (designated *coq1-coq8*) and are nonrespiring, nuclear petite (ρ^+) mutants.[8–10] Together, the Q-deficient mutant strains of yeast and *E. coli* have allowed for the isolation and characterization of many of the genes and polypeptides involved in Q biosynthesis. In general these genes have been recovered through their ability to rescue the respiration deficient phenotype and hence restore growth on nonfermentable carbon sources. The gene/enzyme relationships in the *E. coli* and *S. cerevisiae* Q biosynthetic pathways have recently been reviewed.[11–13] Utilizing the yeast system, it has been possible to isolate and identify corresponding mammalian, plant, and nematode homologues of the yeast *COQ* genes.[14–18] Based on the shared function of yeast, mammalian, plant, and nematode Q biosynthetic genes, *S. cerevisiae* provides an ideal model system for the analysis of this pathway in higher eukaryotes. This review will summarize current work in characterizing the synthesis of 4-hydroxybenzoic acid (4-HB, the aromatic ring precursor of Q), the synthesis and attachment of the polyisoprene tail to 4-HB, and the ring modification steps leading to the fully substituted benzoquinone ring of Q.

12.2 SYNTHESIS OF 4-HYDROXYBENZOIC ACID (4-HB), THE RING PRECURSOR

The aromatic precursor of the benzoquinone ring of Q is 4-HB.[1,2] In microorganisms that synthesize aromatic amino acids, 4-HB is a product of shikimate pathway (Figure 12.2). Species that do not retain the ability to synthesize the aromatic amino acids must generate 4-HB from tyrosine obtained in the diet. This section summarizes studies on the origin of 4-HB in *E. coli*, yeast, animals, and plants.

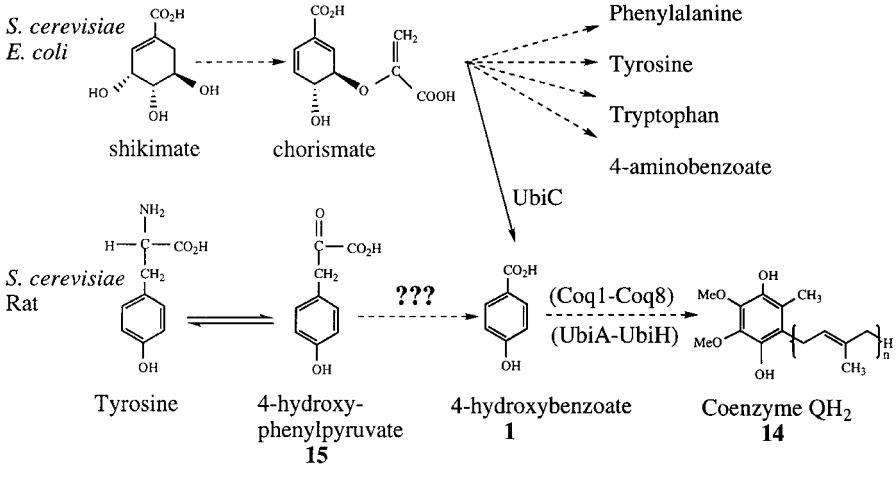

FIGURE 12.2 Metabolic origin of the benzoquinone ring of Q in *S. cerevisiae*, *E. coli* and rat. Wild-type yeast possesses two pathways for the synthesis of 4-hydroxybenzoic acid (4-HB) and normally utilizes chorismate as a precursor. However, an alternate pathway utilizing tyrosine is employed by yeast mutant strains harboring a lesion in the aro pathway prior to chorismate (e.g., yeast *aro1c* mutants lacking 5-dehydroquinate synthetase, or *aro2* mutants lacking chorismate synthase).[23] This latter pathway (designated by ???) is thought to be responsible for synthesis of 4-HB in other eukaryotes that have lost the *de novo* pathways of aromatic amino acid biosynthesis, such as the rat. Solid arrows designate a single step; dashed arrows designate a pathway requiring several steps. (From Clarke, C. F., *Protoplasma*, in press, 1999. With permission.)

12.2.1 UbiC Catalyzes the Exclusive Route to 4-HB in *E. coli*

E. coli strains harboring mutations in the *ubiC* gene are unable to carry out the conversion of chorismate to 4-HB, and are Q-deficient.[19] This analysis of the *ubiC* mutant suggested that the production of Q in *E. coli* depends on this one-step reaction. The *E. coli ubiC* gene was isolated and shown to encode the enzyme chorismate pyruvate-lyase.[20,21] An isotope feeding experiment was performed with [COOH-^{13}C] shikimate and an *E. coli ubiA* mutant strain.[22] Since this Q-deficient mutant is unable to prenylate 4-HB (see Figure 12.1), 4-HB accumulated in the culture media. Recovery of 4-HB and analysis of the ^{13}C-label showed the complete retention of the carboxyl-labeled carbon, consistent with the direct conversion of chorismate to 4-HB. Since a route that involved 4-HB formation from tyrosine (e.g., chorismate $\rightarrow$ tyrosine $\rightarrow$ 4-HB) requires loss of this carboxyl carbon, this experiment showed that formation of 4-HB from tyrosine does not occur to a significant extent in *E. coli.*

12.2.2 Yeast Possess Two Pathways for 4-HB Synthesis

In yeast, 4-HB may be produced directly from chorismate as in *E. coli*, or alternatively, 4-HB may be derived from tyrosine (Figure 12.2).[23] This redundancy of pathways to 4-HB presumably accounts for an absence of yeast mutants defective in producing 4-HB. In labeling experiments with ^{14}C-tyrosine or ^{14}C-shikimate, wild type yeast incorporated 20 times less tyrosine into the benzoquinone ring of Q than shikimate.[23] However, yeast mutants unable to synthesize either shikimate (aro1c mutants), or chorismate (*aro2* mutants) are able to efficiently convert tyrosine to 4-HB, suggesting that this pathway can fully compensate when required.[23] It is possible that the first step in the conversion of tyrosine to 4-hydroxyphenylpyruvate (compound **15**) is mediated by the pyridoxal phosphate-dependent aminotransferase encoded by the *S. cerevisiae ARO8* gene.[24] However, although its role in tyrosine synthesis is well characterized, its role in the degradation of tyrosine is not as clear.

12.2.3 4-HB Synthesis in Animals

In rat liver, both tyrosine and phenylalanine serve as precursors for 4-HB, however, the incorporation of phenylalanine is thought to proceed primarily following its conversion to tyrosine.[25] *In vitro* assays have shown that cell-free extracts of both *S. cerevisiae* and rat liver readily convert tyrosine to **15** (Figure 12.2). This step is dependent on pyridoxal phosphate (vitamin B_6) and there is an interesting correlation between the age-related decline in Q and vitamin B_6 levels in humans.[26] As emphasized in Figure 12.2, the individual steps of the pathway from **15** to 4-HB have not been characterized. The *in vitro* conversion of **15** to 4-hydroxyphenyl-lactate (**16**, Figure 12.3) has been observed in both yeast and rat liver extracts.[23,25] However, it is not clear how *S. cerevisiae* and animal cells convert **16** to 4-HB; three possible routes are indicated in Figure 12.3. The conversion of **16** to 4-coumarate (**17**) followed by shortening of the side chain via β-oxidation is the scheme that is generally assumed to operate in eukaryotes.[2,25] However, experiments with rat liver extracts have not been successful in producing the enzymatic conversion of **16** to **17**.[27] It is presumed that the subsequent β-oxidation steps are either carried out as a part of the general system of fatty acid degradation, or are mediated by specific enzymes.[27]

12.2.4 4-HB Synthesis in Plants

Recently Loscher and Heide[28] have developed an efficient and sensitive assay for 4-HB. This assay employed synthetic ^{14}C-radiolabeled precursors **17** and **20** and soluble, cell-free extracts of cultured *Lithospermum erythrorhizon* plant cells. With this system, the formation of ^{14}C-4-HB was shown to be dependent on ATP, Mg^{2+}, and CoA. The addition of an excess amount of unlabeled intermediates (swamping experiments) suggested that the CoA derivatives are required. Oxidation of **20** to **21** proceeded in a NAD^+-dependent thiolysis reaction, and the CoA esters were rapidly hydrolyzed

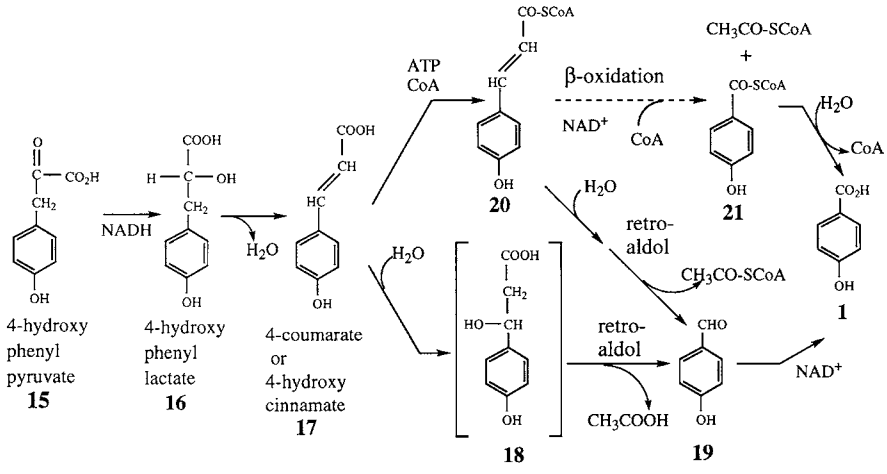

FIGURE 12.3 Proposed pathways for the biosynthesis of 4-HB. Upper pathway, β-oxidation mechanism; lower pathway, retro-aldol mechanism. Recent evidence obtained with plant cell extracts supports the β-oxidation pathway. (Figure adapted from Loscher, R. and Heide, L., *Plant Physiol.*, 106, 271, 1994. With permission.)

to the acids. These results provide strong evidence that 4-HB is generated via the β-oxidation pathway shown in Figure 12.3. Earlier reports suggesting that **19** may be formed from either **17** or **20** by a retro-aldol reaction[29,30] now appear to be an artifact caused by the rapid hydrolysis of the intermediate CoA esters in the cell free extracts.[28] Further work is needed to determine whether this β-oxidation pathway is also employed by other eukaryotic cells.

12.3 MAKING AND ATTACHING THE TAIL: POLYPRENYL DIPHOSPHATE SYNTHASE AND TRANSFERASE

12.3.1 Production of the Isoprenoid Diphosphate Precursors

Dimethylallyl-diphosphate (**2**) and isopentenyl-diphosphate (**3**) provide the building blocks for the synthesis of the polyisoprene diphosphate (**4**) or "tail" of Q (Figure 12.1).[1,2] In *E. coli*, **2** and **3** are derived from pyruvate and glyceraldehyde 3-phosphate via 1-deoxy-D-xylulose-5-phosphate, which is synthesized by the *dxs* gene product.[31,32] Thus, *E. coli*, and presumably most other bacteria, synthesize the tail of Q via the "nonmevalonate" or Rohmer pathway of isoprenoid biosynthesis.[33,34] Interestingly, in plants the Rohmer pathway operates within the chloroplasts (to produce plastoquinone, phytol, and carotenoids), while the classical acetate/mevalonate pathway operates in the cytoplasm and is responsible for sterols and the isoprenoid tail of Q.[35,36] In yeast, fungi, and animals the isoprenoid tail of Q is derived from mevalonate, which is converted to **2**, **3**, geranyl diphosphate, farnesyl diphosphate, and longer polyprenyl diphosphates (**4**) by a series of enzymes that function to supply the cell with isoprene units for both sterol synthesis and the synthesis of nonsterol products.[1,2,37,38] Thus, in these eukaryotic cells the synthesis of Q depends on mevalonate produced by 3-hydroxy-3-methylglutaryl coenzyme *A* reductase, and polyisoprene availability.[39]

12.3.2 Synthesis of the Polyprenyl Diphosphate Tail

Genes encoding the short, medium, and long chain polyprenyl diphosphate synthases have been isolated from a variety of species, and a recent review summarizes the enzymatic properties of these enzymes.[40] The short chain polyprenyl synthases ($n = 2$ to $n = 4$) are designated as Class I enzymes and are responsible for generating biosynthetic precursors such as geranyl-, farnesyl-, and

TABLE 12.1
Polyisoprene Diphosphate Synthases Involved in the Synthesis of Isoprenoid Quinones

Species	$Quinone_n$	Gene	Acc. No.[a]	Synthase	Class	Reference
Saccharomyces cerevisiae	Q6	*COQ1*	JO5547	hexaprenyl	?[b]	43
Micrococcus luteus B-P26	MK_6	*hexsa/hexsb*	AB003187	hexaprenyl	II	44
Bacillus subtilis	MK_7	*gerC1/gerC3*	M80245	heptaprenyl	II	45
Bacillus stearothermophilus	MK_7	*heps1/heps2*	D49976	heptaprenyl	II	46
Haemophilus influenzae	MK_7	*ispB*	H10881	heptaprenyl	III[c]	47
Escherichia coli	Q_8, MK_8	*ispB*	U18997	octaprenyl	III	48
Rhodobacter capsulatus	Q_9/Q_{10}	*sdsA*	AB001997	solanesyl decaprenyl	III[c]	49
Synechocystis sp. PCC6803	PQ_9	*ispB*	D90899	solanesyl	III[c]	47
Schizosaccharomyces pombe	Q_{10}	*dps*	D84311	decaprenyl	?[b]	50
Gluconobacter suboxydans	Q_{10}	*ddsA*	AB006850	decaprenyl	III[c]	51

[a] GenBank Accession Number.
[b] Class unknown; expression of gene in *E. coli* did not result in active enzyme.
[c] Likely to be class III; expression of gene in *E. coli* or *S. cerevisiae* resulted in active enzyme.

geranylgeranyl-diphosphate. The Class I enzymes function as tightly associated homodimers, requiring only a divalent metal cation such as Mg^{2+} or Mn^{2+} as a cofactor. The short chain polyprenyl diphosphates serve as substrates for the Class II and Class III enzymes, which generate medium and long chain polyprenyl diphosphates. Table 12.1 summarizes the gene-enzyme relationships for both Class II and Class III synthases. In general, the Class II enzymes function as heterodimers, with subunits that readily dissociate under physiological conditions.

Characterization of the two components from *Micrococcus luteus* B-P26 showed each subunit (A and B) to be inactive individually, but upon incubation with Mg^{2+} and farnesyldiphosphate, form an active complex (AB). Addition of IPP to the active complex resulted in formation of the products, hexaprenyl diphosphate (**4**) and PPi, and also resulted in the dissociation of the complex into the components A and B.[40] This class of enzymes has so far been identified only in gram positive bacteria, which do not produce Q, but instead synthesize the prenylated napthoquinone, menaquinone, (MK_n) (Table 12.1).[41] Purification and characterization of the solanesyl diphosphate synthase from *M. luteus* (an isolate distinct from *M. luteus* B-P26) showed it to be active as a homodimer, and defined the Class III polyprenyl diphosphate synthases.[42] The assignment of many of the long chain polyprenyl diphosphate synthases as Class III in Table 12.1 is still tentative, and is based mainly on the ability of single genes to restore this activity when expressed in either *E. coli* or yeast mutants known to be defective in this step. Amino acid sequence comparison shows that Class I and III, and the second (or B) subunit of Class II polypeptides, each share 7 conserved regions. A combination of site directed mutagenesis and structural studies indicate that aspartate-rich regions II and VI are especially important for catalysis.[40]

12.3.2.1 Polyprenyl Diphosphate Synthase Determines the Tail Length of Q

It is now clear that polyprenyl diphosphate synthase enzymes are responsible for determining the number of isoprene units used in the assembly of the tail of Q (designated as *n* in Figures 12.1–12.3). The role of these enzymes in determining the tail length of Q was convincingly demonstrated by expressing different homologues of genes encoding polyprenyl diphosphate synthases in either *E. coli* or *S. cerevisiae* mutant strains harboring a null mutation in the *ispB* or *COQ1* gene, respectively.[47,49,51–53] In these experiments, both Q-deficiency and growth on a nonfermentable carbon source were rescued. A variety of Q isoforms were produced in which the length of the polyisoprene

tail ranged from $n = 5$ to $n = 10$, depending on the distinct polyisoprene diphosphate supplied by each homologue. From these studies it is apparent that the series of subsequent enzymes required to produce Q tolerate substrates containing a wide range of tail lengths, and that the different isoforms of Q so produced each functionally replace the endogenous Q.

Attempts to rescue the *E. coli ispB* mutant with the *S. cerevisiae COQ1* gene were not successful.[54] Similarly, the expression of the *S. pombe dps* gene in either *E. coli* or *S. cerevisiae* failed to restore Q biosynthesis.[50] This failure to rescue is not due to the type of Q isoform produced, because expression of the *ddsA* gene from *Gluconobacter suboxydans* rescued both *E. coli ispB* mutants and *S. cerevisiae coq1* mutants for growth on nonfermentable carbon source and resulted in Q_{10} biosynthesis.[51,53] Instead, the inability to detect activity when these yeast genes are expressed in heterologous systems suggests that the yeast enzymes are probably not active as Class III-type enzymes, and that additional factors are needed for activity. The purification and characterization of the polyprenyl diphosphate synthases from yeast and other eukaryotic cells constitute important studies that may reveal a two component system similar to that of the Class II enzymes, or may perhaps identify yet another class for the polyprenyl diphosphate synthase enzyme family.

12.3.2.2 ispB is an Essential Gene in *E. coli*

The unusual observation was made that the ispB gene is an essential gene in *E. coli*.[47] It is required for aerobic growth, even on media containing a fermentable carbon source, such as glucose. Such mutants required that the *ispB* gene (or a homologue) be present on a plasmid, even during growth in rich media.[47] This phenotype is surprising because other genes required for Q biosynthesis in *E. coli* (the *ubi* genes) are not essential for growth, though they are required for growth on media containing succinate as the sole carbon source.[13] It is possible that the strict requirement for the *ispB* gene in *E. coli* may stem from its function in both Q and menaquinone/demethylmenaquinone production. Studies by Wallace and Young[55] indicated that *ubiA*$^-$/*menA*$^-$ double mutants (lacking the polyprenyl diphosphate:ring transferase activities required for production of Q, menaquinone, and demethylmenaquinone), were unable to grow aerobically. This result suggests that either *E. coli* cannot grow aerobically solely using glycolysis, or perhaps may require these quinones for other metabolic pathways essential to growth.

12.3.3 Attachement of the Polyprenyl Tail to 4-HB

The polyprenyl diphosphate:4-HB transferase generates the Q-intermediate, 3-polyprenyl-4-hydroxybenzoic acid (**5**, Figure 12.1). The *S. cerevisiae* and the *E. coli* genes encoding this enzyme have been isolated and characterized as *COQ2* and *ubiA*, respectively.[21,22,56–58] Expression of the *S. cerevisiae COQ2* gene rescued the respiration defective phenotype and Q-deficiency of an *E. coli ubiA* mutant strain, indicating a conservation of function.[59] The predicted amino acid sequences of *ubiA* and *COQ2* contain two conserved domains found in a family of polyprenyl-transferases and six potential membrane spanning domains.[56,60] The *E. coli ubiA* gene has been overexpressed and the membrane associated polyprenyl diphosphate:4-HB transferase activity was enriched 3000-fold.[60] This enzyme preparation accepted a broad array of polyprenyl diphosphate substrates, including $n = 2$, $n = 3$ and $n = 9$. A similar lack of specificity was observed in assays of *S. cerevisiae* cell extracts, however, the substrate specificity is influenced by the Mg^{2+} concentration in the assay buffer; at 3 mM the enzyme is relatively specific for the endogenous polyprenyl diphosphate, while at 33 mM Mg^{2+}, the enzyme makes use of any available allylic polyprenyl diphosphate as substrate.[56]

Although mammalian *COQ2* homologues have not been described, *in vitro* assays indicate the presence of polyprenyl diphosphate:4-HB transferase activity in mitochondria isolated from rat heart and liver.[61,62] However, other studies in rat liver detect polyprenyl diphosphate:4-HB transferase activity in the endoplasmic reticulum and Golgi subcellular fractions.[63–65] It seems likely that the

precise subcellular localization(s) of this activity, and indeed of Q synthesis in general, will require localization of the polypeptide components (see Section 12.4.1). The polyprenyl diphosphate:4-HB transferase activity in rat heart and liver mitochondria preparations was also able to prenylate 3,4-dihydroxy benzoate and 3-methoxy-4-hydroxy benzoate.[62] The efficiency of prenylation of these latter aromatic rings was nearly as efficient as that of 4-HB, and ranged from 30 to 80%. This finding indicates that there may be alternate routes in the Q biosynthetic pathway, since prenylation of these intermediates generates compounds **8** and **9** (Figure 12.1). This intriguing observation also suggests that a variety of aromatic head groups may compete with 4-HB for prenylation. Hamilton and Cox[66] have proposed that such prenylation probably accounts for the growth inhibition of 5 μM 4-aminobenzoic acid on *E. coli* strains, and the reversal of such inhibition by 50 μM 4-HB.[67] The prenylation of 4-aminobenzoic acid (forming the product 3-polyprenyl-4-aminobenzoate) has been observed in aromatic auxotrophic mutants of *E. coli* and in mammalian tissues.[66,68]

12.4 RING MODIFICATION STEPS

The proposed order of ring modification steps in Q biosynthesis is shown in Figure 12.1. Support for the divergence between *E. coli* and yeast derives from the isolation of compound **6** in *ubiB* deficient *E. coli* mutants,[69] compound **8** in *coq3* mutants of *S. cerevisiae*,[5] and compound **9** in another *S. cerevisiae* mutant.[6] Gibson and Young[4] analyzed other *E. coli* mutants and characterized *ubiH*, *ubiE*, *ubiF*, and *ubiG* mutants as accumulating compounds **10**, **11**, **12**, and **14**, respectively. Thus, the *E. coli* Q biosynthetic pathway as depicted in Figure 12.1 is supported by the identification of specific intermediates accumulating in each Q-deficient ubi mutant. However, the order of steps in the yeast pathway presented in Figure 12.1 is more speculative, as only a few of the intermediates shown have been recovered from mutant *coq* strains. In fact, our analysis of *coq* mutant strains (*coq3–coq8*) shows that compound **5** accumulates as the single predominant intermediate in each.[70,71] This observation, coupled with recent genetic evidence of possible interactions between the *COQ* gene products, suggests that a multisubunit complex may be involved in the conversion of **5** to QH_2.

12.4.1 Mitochondrial Localization of Yeast Coq Polypeptides

Table 12.2 summarizes our knowledge regarding the subcellular locations of the Coq polypeptides in yeast. Inspection of the predicted amino acid sequences of the Coq polypeptides shows that, with the exception of Coq7, the Coq polypeptides contain typical mitochondrial leader sequences (e.g., the amino-terminal residues are relatively rich in positively charged amino acids, lack acidic residues and form amphipathic α-helices).[72,73] In addition Coq2p, Coq3p, Coq4p, Coq5p, Coq6p, and Coq8p contain a 3-amino acid consensus motif, which has been identified as being common to leader sequences of mitochondria matrix polypeptides.[74] For seven of the eight Coq polypeptides, *in vitro* import and processing has been demonstrated and is dependent on a mitochondria membrane potential (Table 12.2). In addition, most of the Coq polypeptides have also been localized to mitochondria by subcellular fractionation studies. These data indicate that in yeast, biosynthesis of Q is performed by the mitochondria. It is apparent there must be intracellular transport of Q, since it is present in the plasma membrane of yeast.[85,86] The mechanisms accounting for this transport have not been studied.

12.4.2 Monooxygenases and Hydroxylases

Yeast *coq6* mutants fail to synthesize Q and accumulate **5** as the predominant Q-intermediate (Figure 12.1).[71] Two independent yeast genomic clones were recovered that rescue the *coq6* mutant.[87] Each clone encodes a polypeptide that is homologous to various aromatic hydroxylases, including the *E. coli UbiH* polypeptide, suggested to catalyze quinone formation by converting compound **10** to **11** in a monooxygenase oxidation step.[88,89] Based on this homology (Figure 12.4) it was thought that the polypeptide encoded by the *COQ6* gene might catalyze the hydroxylation

TABLE 12.2
Mitochondrial Localization of Yeast Coq Polypeptides and Analysis of their Leader Sequences

Polypeptide	N-Terminal Sequence	Localized to Mitochondria by: *in vitro* Import	Localized to Mitochondria by: Subcellular Fractionation
Coq1	MFQ**R**SGAAHHI**K**LISS**RR**C**R**F**K**SSFAVALN	N.D.	N.D.
Coq2	MFIWQ**RK**SILLG **R** S I LG S G**R**V TV↑AGIIGSS**RKR**YT	+[75]	N.D.
Coq3	MGFIMLL**RSR**FL**K**VIHV**RK**QLSACS **R** F A IQ T QT**R**C**K**ST ↑	+[76]	+[80]
Coq4	ML**R**LSLL**R**STLTLPV**K**CQ**R** **R** G L IL P AAAM↑Y	+[79]	+[83]
Coq5	MLISS**R**IV**R**SSLVNVPL**R**LS **R** C F TQ A H**R**AC**K**↑	+[77]	+[77,81]
Coq6	MFFS**K**VMLT**R** **R** I L V**R** G LATA**K**SSAP**K**L ↑	+[79]	+[84]
Coq7	MFPYFY**RR**EFYSCENVVIFSS**K**PIQGIKIS (−) (−)	+[78]	+[82]
Coq8	MVTNMV**K**L**R**NL**RR**LYCSS**R**LL **R** T I QN G **R**IS SVSS ↑	+[79]	+[84]

Note: N.D., not done; **K, R,** Positively charged residues; (−), negatively charged amino acids.

↑ Indicates site of mature amino terminus of Coq polypeptide as predicted by the 3 amino acid motif (underlined residues) present in leader sequences of polypeptides targeted to the mitochondria matrix compartment (Hendrick et al., 1989). References for *in vitro* import and for subcellular fractionation are indicated by the bracketed numbers.

step in Q biosynthesis, which generates the quinone moiety in eukaryotic cells. However, other hydroxylation reactions are possible as well. There are three hydroxylation events in Q biosynthesis, and in fact, an ORF in *E. coli* has been recently identified as a candidate for the *ubiF* gene, which also shares a great deal of identity with Coq6p.[90] Over the entire polypeptide sequence, Coq6p and UbiHp are 25% identical while Coq6p and UbiFp are 24% identical (Figure 12.4). Coq6p, UbiHp, and UbiFp each share identities with a large family of proteins that function as flavoprotein monooxygenases.[91] These polypeptides each contain a consensus sequence for ADP-binding (Region I),[92] a fingerprint sequence proposed to play a dual role in binding both FAD and NAD(P)H (Region II),[93] and a third fingerprint consensus sequence for binding to the ribityl moiety of FAD (Region III).[94] The presence of these three sequence motifs in Coq6p, UbiHp, and UbiFp suggest that each functions as a flavoprotein monooxygenase, a prediction that can now be tested directly by using *in vitro* assays with candidate substrates (**10** and **12**, Figure12.1) and coenzymes FAD and NADPH. These assays should make clear the true function of the polypeptides encoded by the *COQ6*, *ubiH*, and *ubiF* genes.

The *E. coli ubiB* gene is required for a monooxygenase step and accumulates intermediate **6**.[69,95] This gene has yet to be identified. The *fre* gene has been proposed to be a candidate *ubiB* gene.[13,96] However, our analysis of a *fre* disruption mutant, *E. coli* LS1312, *fre::kan*[97] showed it produced normal levels of Q_8 and therefore is not required for Q biosynthesis.[84]

12.4.3 Decarboxylation

E. coli contains two genes, *ubiD* and *ubiX*, that are involved in the decarboxylation of **5**.[13] A *ubiD* mutant strain accumulated **5**, and *in vitro* assays showed a complete absence of 5:decarboxylase activity.[69,98] However, the *ubiD* mutant was leaky since Q was produced, although at only 20% of normal levels.[69] The *E. coli ubiD* gene has not yet been characterized, though its map position is close to that of *ubiE*, the C-methyltransferase in the pathway.[69,96,99] A second gene

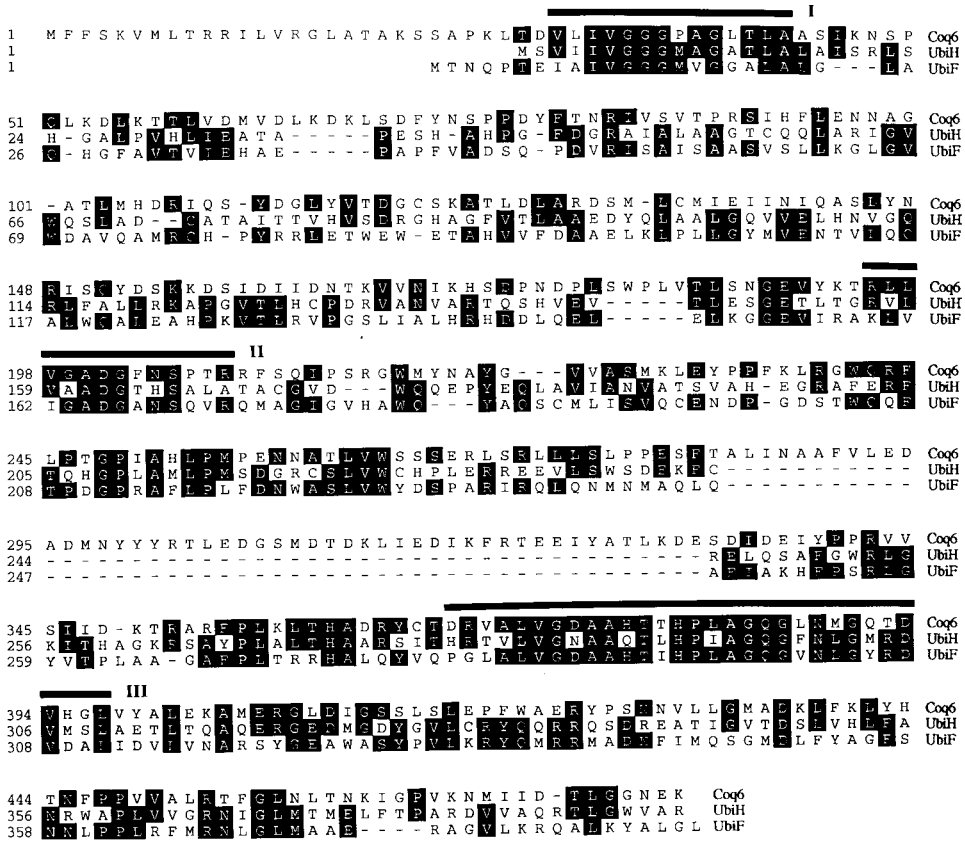

FIGURE 12.4 Alignment of predicted yeast *COQ6* amino acid sequence with two *E. coli* homologues and identity of sequence motifs found in FAD-binding aromatic hydroxylases. The sequence of the yeast Coq6 polypeptide (GenBank AF003698) is shown in alignment with *E. coli* UbiH (2-octaprenyl-6-methoxyphenol hydroxylase, GenBank D90281) and with a recently identified candidate gene for *E. coli* UbiF (GenBank ECAE000170; Felkai et al., 1999). Alignments were created on DNASTAR™'s Megalign with the Clustal method and the PAM 250 residue weight table. Identical amino acid residues are shaded. The solid bars designate three motifs found in a large family of flavin-dependent monooxygenases: Region I is an ADP binding fingerprint identified by Wierenga et al.;[92] Region II is implicated in the recognition of NADH or NADPH and is also involved indirectly in binding the pyrophosphate moiety of FAD;[93] Region III contains a consensus sequence for binding to the ribityl moiety of FAD.[94]

encoding a related decarboxylase function, namely *ubiX*, is thought to account for the residual 20% of Q biosynthesis in the *ubiD* mutant strain. The *ubiX* gene, also named *dedF*, was first identified in a *Salmonella typhimurium* mutant strain that was phenotypically similar to the *ubiD E. coli* mutant.[100] Recently, the *E. coli ubiX* gene was recovered through its ability to restore synthesis of Q when overexpressed in a thiol-hypersensitive mutant strain of *E. coli*.[101] Such thiol hypersensitivity in this mutant strain probably results from the requirement for Q (and an intact respiratory chain) in the introduction of disulfide bonds via the DsbA and DsbB system in the periplasm of *E. coli*.[102,103] This thiol hypersensitivity is also seen in *ubiA* mutants. It is likely that the thiol hypersensitive strain was a double mutant—the lesion in *ubiX* was verified and a second lesion in *ubiD* was postulated.[101] It is interesting that a homologue of *ubiX* exists in *S. cerevisiae*, which has been identified as the *PAD1* gene (48% amino acid sequence identity).[104] The *PAD1* gene product is required for the *in vitro* decarboxylation of a variety of phenylacrylic acid substrates,

including 4-hydroxycinnamate (**17**, Figure 12.3). It is not yet known whether a *pad1* yeast mutant is deficient in producing Q.

12.4.4 O-Methyltransferase

12.4.4.1 Two O-Methylation Steps are Catalyzed by One Enzyme

Both *E. coli* and yeast genes encoding O-methyltransferase enzymes in Q biosynthesis were isolated by their ability to restore respiration and Q biosynthesis in *ubiG* and *coq3* mutants, respectively.[105,106] The predicted amino acid sequences of UbiGp and Coq3p are 40% identical, and both contain four sequence motifs (I, post I, II, and III) conserved in a large family of methyltransferase enzymes that use S-adenosylmethionine (AdoMet) as a methyl donor (Figure 12.5).[107,108] An *E. coli ubiG* mutant strain (AN151) was unable to carry out the last O-methylation step[110] and therefore accumulated compound **13**, the last intermediate in Q biosynthesis.[111] Analysis of the *coq3* mutant yeast suggested that it is defective in the first O-methylation step, since compound **8** was found to accumulate.[5]

A cross-species comparison combined with *in vitro* assays now indicate that both O-methylation steps are catalyzed by the same enzyme.[76,80,112] The *in vitro* O-methylation assays have employed synthetic farnesylated analogs of **7**, **8**, and **13** as substrates, and rely on the detection of transfer of a radiolabeled methyl group from AdoMet to a product that coelutes with farnesylated **10**, **9** or **14**, respectively. Such assays were performed with isolated yeast mitochondria,[80,112] cell free extracts of *E. coli*,[76] or the purified UbiG polypeptide,[80] and indicate that the presence of Coq3 or UbiG is required in order to observe *in vitro* O-methylation, and that both Coq3 and UbiG recognize all three substrates. The accumulation of **13** in the original *E. coli ubiG* mutant strain is due to the presence of a leaky allele of *ubiG*; this strain is able to methylate the early intermediate (**7**) although with a greatly decreased activity.[76] In contrast, a strain containing a disruption of the *ubiG* gene lacks both O-methyltransferase activities.[76] It is curious that in this *ubiG* disruption mutant, **6** was detected as an accumulating intermediate, but not **7**.

The promiscuous O-methylation of substrates is also borne out by the ability of the *E. coli ubiG* gene to restore respiration and Q biosynthesis in the yeast *coq3* mutant.[76] Such rescue requires that the UbiG polypeptide contain an amino terminal mitochondria import leader sequence. Thus the mitochondria targeting of the UbiG O-methyltransferase is an essential feature of the ability to restore respiration and Q biosynthesis in yeast *in vivo*. Subcellular localization of the yeast Coq3 polypeptide shows that it is present in mitochondria, where it is peripherally associated with the matrix-side of the inner membrane.[80] *In vitro* mitochondria import reactions show that the Coq3 polypeptide is proteolytically processed and imported by yeast mitochondria (Table 12.2).[76] Thus, both the first and last O-methylation steps in the yeast Q biosynthetic pathway occur within the mitochondria compartment.

12.4.4.2 Mammalian and Plant *COQ3* cDNAs Rescue the Yeast *coq3* Mutant

A rat *COQ3* cDNA was isolated based on its ability to restore growth of the yeast *coq3* mutant on a nonfermentable carbon source.[14] The deduced rat amino acid sequence has a 39% identity over 138 amino acids with the yeast Coq3 O-methyltransferase. The N-terminal sequence is rich in basic residues, and shares other features in common with mitochondria import sequences. An *Arabidopsis COQ3* homologue (AtCOQ3) was also cloned by functional complementation of the yeast *coq3* mutant.[17] Its predicted amino acid sequence was 38% and 26% identical with rat and yeast Coq3p, respectively. Western blot analysis of both *Arabidopsis thaliana* and pea protein extracts indicated that the polypeptide was localized to mitochondria membranes. Recently a full length human Coq3 cDNA has been recovered (through its homology with the rat cDNA) which also restores respiratory function, methyltransferase activity, and Q biosynthesis to the yeast *coq3* mutant when expressed in multicopy.[84] It is clear that this enzyme has a wide substrate specificity; even the higher eukaryotic

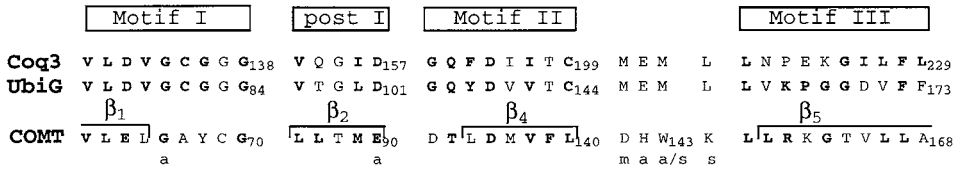

FIGURE 12.5 Alignment of the *S. cerevisiae* Coq3, *E. coli* UbiG and rat COMT amino acid sequences across methyltransferase motifs I, post-I, II and III. The four motifs of sequence similarity present in AdoMet-dependent methyltransferases are shown; bold residues designate a match with the consensus amino acids identified by Kagan and Clarke.[107] The carboxy-terminal residue of each motif is numbered and indicates the position of the motifs relative to the linear amino acid sequence of the polypeptide chain. The secondary structure elements in the crystal structure of the rat soluble COMT (β_1, β_2, β_4, and β_5) and the important active site residues involved in the binding of ligands are indicated. a, AdoMet; m, magnesium; s, substrate.[109]

forms including rat and human can methylate both eukaryotic substrates and the distinct prokaryotic substrate in *in vitro* assays. This type of promiscuous activity has also been seen in a related enzyme, catechol-O-methyltransferase (COMT). COMT has many physiologically relevant substrates including dopa, catecholamines, their hydroxylated metabolites, and many other analogues.[113] The COMT, Coq3, and UbiG enzymes all required a divalent cation, but comparison of their amino acid sequences fails to reveal any homology outside of the AdoMet-dependent methyltransferase motifs (Figure 12.5). Based on the amino acid identities within the motif regions, and the similar catechol structure of the substrates, it seems likely that the O-methyltransferase in Q biosynthesis may rely on a similar mechanism to that of COMT. In fact, many COMT inhibitors have been developed as potential adjuncts in the drug therapy of Parkinson's disease.[113] The extent to which the COMT inhibitors affect the Coq3-mediated O-methyltransferase steps in Q biosynthesis has not been investigated. This is a potentially important point since the therapeutic efficacy of Q supplementation is currently being tested in patients with Parkinson's disease.

The above observations suggest that yeast provides an excellent prototype of an eukaryotic Q biosynthetic pathway, and that it can be used to answer fundamental questions about Q biosynthesis in higher eukaryotes. The rat *COQ3* cDNA was used to map the mouse *COQ3* gene to the proximal region of chromosome 4, 3.7 ± 2.6 cM proximal to the marker D4Mit4.[114] Interestingly, this map position places it in close proximity to the mouse *vacillans* or *vc* mutation. *Vacillans* mice were smaller than normal mice and exhibited half the muscle strength of normal mice, decreased fat and muscle mass, decreased mental acuity and reaction times, were easily tired, and sank in swim tests.[115] These symptoms are strikingly similar to those reported for human Q deficiencies. In humans, Q_{10} deficiency has been documented in two sisters with the following symptoms: abnormal fatigability, muscle weakness, learning disabilities, decreased stature, and central nervous system dysfunction.[116] More recently, a severe Q_{10} deficiency was detected in a four-year-old boy who presented with muscle weakness, seizures, and elevated levels of lactate in cerebrospinal fluid.[117] The amounts of coenzyme Q in muscle mitochondria were 6% of normal levels and activities of respiratory complexes requiring Q were dramatically decreased. In each reported case, the Q depletion was tissue specific, since there was no impairment detected in cultured fibroblasts or in lymphoblastoid cell lines, and oral administration of Q_{10} resulted in significant improvement in physical performance.[116,117] One of the most intriguing findings that has emerged from the study of these patients is the implication of muscle specific expression of one or more Q biosynthetic enzymes. Certainly the *COQ* gene family (*COQ1–COQ8*) as identified in the yeast collection constitute candidate genes for these human Q deficiency diseases, although increased turnover of Q has not yet been ruled out.

12.4.5 C-Methyltransferase

E. coli ubiE mutants are deficient in the C-methylation step of Q biosynthesis[95] and in the C-methylation of demethylmenaquinone to form menaquinone.[118] Thus, the ubiE gene was hypothesized to correspond to a C-methyltransferase.[95] Lee et al.[99] identified an ubiE candidate gene, referred to as o251, based on its location in a region of the *E. coli* chromosome corresponding to the reported genetic map position of ubiE (86 min)[95,96] and the presence of the methyltransferase motifs identified by Kagan and Clarke.[107] Several lines of evidence confirmed the identity of o251 (GenBank M87049) as *ubiE*. The expression of o251 in AN70 (an *E. coli* strain containing the ubiE401 mutation) restored both the growth of this strain on succinate and its ability to synthesize Q_8. Disruption of o251 in a $ubiE^+$ parental strain produced a mutant with a phenotype similar to that of AN70, including defects in growth on succinate, in Q_8 synthesis, and in the accumulation of compound **11** and demethylmenaquinone.[99] The mutation present in AN70 was identified as a substitution of Asp for Gly_{142}. This substitution occurs in a highly conserved region of the polypeptide, and maps to residues immediately following the methyltransferase motif II (equivalent to W_{143} in COMT in Figure 12.5). This region has been identified as making contacts with both AdoMet and the catechol substrate in the catechol O-methyltransferase.[109]

A search of the gene databases identified a UbiE homologue in *S. cerevisiae* which we now know corresponds to Coq5 (40% identity over 239 aa). The *COQ5* gene (GenBank P49017) was recovered by functional complementation (restoration of growth on glycerol) of a coq5-1 yeast mutant.[77,81] The cloned gene is allelic with the original coq5-1 yeast mutant, and the polypeptide product colocalizes with the matrix of yeast mitochondria. The yeast *COQ5* gene product is required to observe C-methylation of a farnesylated analog of **11** to **12** *in vitro* with isolated yeast mitochondria.[81] Similar assays with *E. coli* extracts show that C-methylation of **11** to form **12** is dependent on an intact ubiE gene. Finally, the yeast *COQ5* gene restores succinate growth in the AN70 ubiE mutant strain,[77] and the *E. coli* ubiE gene (expressed from a yeast CYC1 promoter and containing a mitochondria leader sequence) rescues growth on glycerol of several yeast strains harboring mutations in coq5.[81] Thus it is likely that each polypeptide functions as the C-methyltransferase in Q biosynthesis; however, direct demonstration of this will require preparations of each of the pure polypeptides.

12.4.6 Unassigned Polypeptides

12.4.6.1 *E. coli* YigR, and *S. cerevisiae COQ4* and *COQ8* Are Required for Q Biosynthesis

There are genes that are known to be required for Q biosynthesis in *E. coli* and *S. cerevisiae*, but for which functions have yet to be assigned. Included in this category is the *E. coli yigR* gene,[119] and the *COQ4* and *COQ8* genes in yeast.[10] As with the other Coq polypeptides, Coq4p and Coq8p have been localized to the mitochondria (Table 12.2). Although it is tempting to speculate that these genes may correspond to either monooxygenases or carboxylases as mentioned above, the amino acid sequences of these gene products do not contain significant homology with any known class of monooxygenase or hydroxylase family of enzymes. Our studies have shown that the *COQ8* gene is identical to the *ABC1* gene.[120,121] Brassuer et al.[122] found that the phenotype of an *abc1* mutant appeared to be similar to that of quinone-deficient yeast and that multiple respiratory complex activities were thermosensitive. It was suggested that the *ABC1* gene product acts like a chaperone, essential for the proper conformation and functioning of the bc_1 complex and that the resultant effects on the other complexes may result from interactions with the modified bc_1 complex. Our recent work shows that the *abc1*/*coq8* mutant is in fact Q-deficient and that both its respiratory deficient phenotype and bc_1 complex activity is rescued by the addition of Q_6 to liquid growth media.[121] These results indicate that the pleiotropic defects in this mutant are due to a lack of Q rather than a specific defect in the bc_1 complex.

12.4.6.2 Yeast *COQ7/CAT5* Functions in Q Biosynthesis

Like the other yeast *coq* mutants, *coq7* mutants lack Q, are respiration defective, and are incapable of growing on nonfermentable carbon sources.[10,123] A yeast mutant harboring the *coq7-1* allele (encoding the substitution of Asp for Gly_{104}) was found to accumulate both **5** and a small amount of **12**.[123] However, *coq7* null mutants produce only **5**, the predominant compound that accumulates in yeast mutants with deletions in any one of six *COQ* genes (*COQ3–COQ8*).[70,71] Transformation of either the *coq7-1* point mutant or the coq7 null mutant with the yeast *COQ7* gene restored both growth on nonfermentable carbon sources and the synthesis of Q. These results support a proposal that Coq7p provides a component of a multisubunit enzyme complex that is required for the conversion of **5** to Q.[71,123]

The yeast *COQ7* gene was independently isolated as *CAT5*, a gene required for the release of gluconeogenic genes from glucose repression.[124] Glucose repression is a global regulatory system in *S. cerevisiae* that affects the transcription of genes involved in gluconeogenesis and respiration.[125] Upon deletion of *CAT5*/*COQ7*, binding of gene activators to the UAS-elements (upstream activating sequences) within gluconeogenic promoters was abolished resulting in a total loss of gluconeogenic gene activation.[124] These data provided support for a role of Cat5p/Coq7p in the cascade regulating gluconeogenic gene activation.

The apparent dual function of Coq7p/Cat5p in yeast Q biosynthesis and glucose derepression raised the question of whether the observed defect in Q biosynthesis resulted from a defect in glucose derepression, or vice versa. Recently, Coq7p/Cat5p has been shown to be a mitochondria protein directly involved in Q biosynthesis, and that the defect in gluconeogenic gene activation in *coq7*/*cat5* null mutants is a secondary effect resulting from a defect in respiration.[82] Moreover, the growth defect of a *coq7*/*cat5* mutant under nonfermentable conditions can be restored by the addition of Q_6 to the media. Such Q_6 supplementation also restores the ability to activate gluconeogenic and respiratory enzymes during the transition from glucose- to ethanol-metabolism.[82] These rescue data clearly restrict the Coq7p/Cat5p function to Q biosynthesis.

12.4.6.3 The *C. elegans clk*-1 Life Span Extension Gene is a Homologue of Yeast *COQ7*

Recently the *C. elegans clk-1* gene was characterized and found to be a homologue of the yeast *COQ7* gene.[18] Specific *C. elegans clk-1* mutants exhibit a pleiotropic phenotype, characterized by delayed embryonic and post embryonic development, a slowing of adult behaviors such as swimming, pharyngeal pumping, and defecation, and an extended life span.[126] The *clk-1* mutants also have an increased resistance to UV- and heat-induced stress.[127,128] This gene is conserved among eukaryotes, including humans, rodents, and the yeast *S. cerevisiae*,[18] but has also been identified in the obligate intracellular parasite *Rickettsia prowazekii*, which, interestingly, is more closely related to mitochondria that any other microbe studied thus far (Figure 12.6).[129] It is interesting to note that the *C. elegans* genome contains homologues of all of the yeast *COQ* genes (*COQ1–COQ8*).[84] The *COQ7* homologues from *C. elegans*, rat, and human are capable of rescuing the yeast coq7 mutant for growth on nonfermentable carbon sources, suggesting a conservation of function and location from yeast to animals.[15,16,18] Our previous results obtained in the *coq7* yeast mutant suggest that the effects on life span and biological timing in *C. elegans clk-1* mutants may relate to changes in the amount or distribution of Q. To investigate this idea, yeast equivalents of the three clk-1 mutant alleles, *coq7-e2519*, *coq7-qm30*, and *coq7-qm51* (Figure12.6) were generated and expressed in both multicopy and single-copy in yeast *coq7* null mutants. Each of the mutant alleles was tested for the ability to restore growth of a yeast *coq7* null mutant on media containing a nonfermentable carbon source (glycerol). We found that rescue of growth on glycerol was both strain-dependent and influenced by the copy number. Four separate *coq7* null strains (prepared in different laboratory "wild-type" backgrounds) gave the following results: in two strains none of

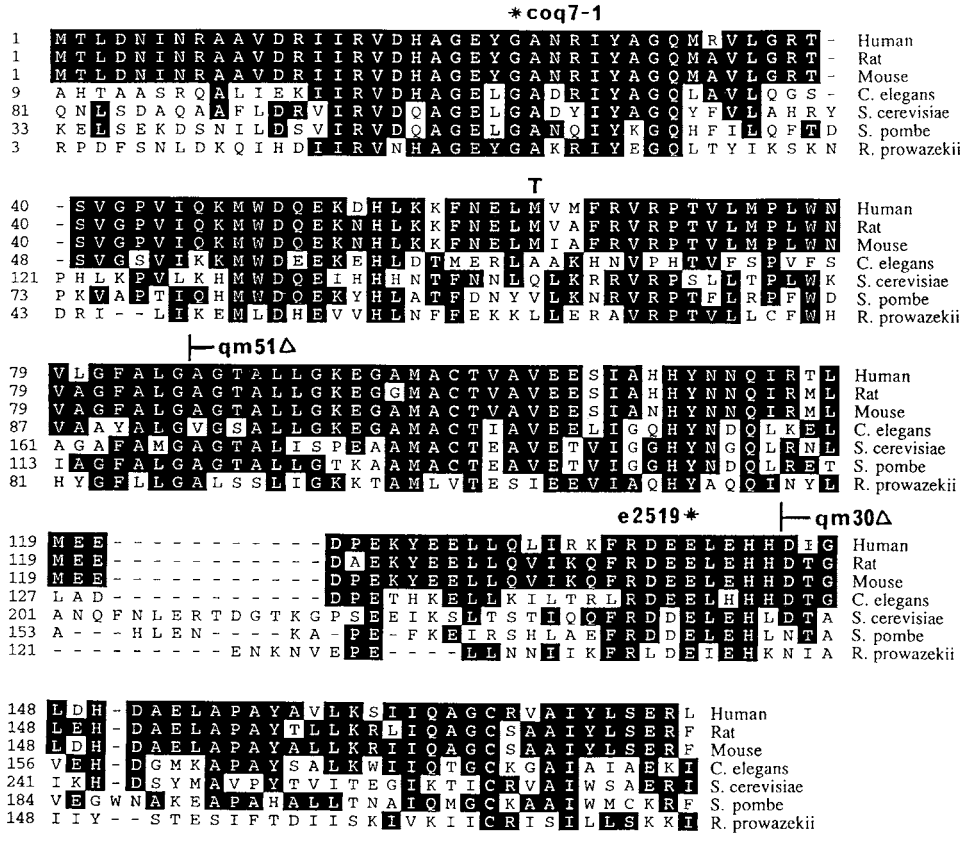

FIGURE 12.6 Amino acid sequence comparison of Coq7/CLK-1 homologues. Alignment of the predicted Coq7 protein sequences of the human (U81276, GenBank), rat (U46149, GenBank), mouse (AF054770, GenBank), *C. elegans* (U13642, GenBank), *S. cerevisiae* (X82930, GenBank), *S. pombe* (CAA21285, GenBank), and *R. prowazekii* (CAA14656) was done using the Clustal method in megalign of DNASTAR. Amino acid residues shared by all of the polypeptides are shaded, introduced gaps are designated with dashes. The Met_{63}/Thr polymorphism in human CLK-1 is noted by a T over the M at position 63. An α-helical transmembrane region is predicted for human CLK-1, residues 73-91, based on the PredictProtein program and is also predicted for each of the other homologues in the corresponding region. Also identified are the *S. cerevisiae coq7-1* allele[123], and three *C. elegans clk-1* mutations.[18] (Figure from Vajo et al., Mammalian Genome, 10, in press, 1999. With permission.)

the mutant alleles were able to rescue, in the third strain only the *coq7-e2519* and *coq7-qm30* alleles rescued, and the fourth was rescued by all three mutant alleles. Log phase cultures of this fourth strain, when grown on media containing glucose and harboring either the *coq7-qm30* or the *coq7-e2519* alleles, produced only 30% and 50% as much Q as the control strain expressing a wild-type *COQ7* gene.[130] These studies indicate that the *clk-1* mutant alleles may be considered to represent either "null" or "partial loss of function" mutations, depending on the background of the yeast strain in which they are expressed. These studies in the yeast system indicate that these mutations may either completely prevent or profoundly decrease the biosynthesis of Q.

However, other investigators studying the nematode system have concluded that the *clk-1* mutations do not profoundly affect Q biosynthesis. Felkai et al.[90] studied mitochondria activity in the three *clk-1* mutant strains of *C. elegans* via two indirect assays: the uptake of G6-rhodamine (a dye that accumulates in mitochondria as a function of the membrane potential), and succinate cytochrome *c* reductase activity. They found that the *clk-1* mutant worms showed only slight

impairment of respiratory function. Addition of Q_1 to mitochondria isolated from the clk-1 mutants produced only a very modest elevation of succinate cytochrome *c* reductase activity, similar to that observed with preparations of wild-type mitochondria. Similarly, oxygen consumption rates were only slightly lower in the *clk-1 e2519* mutant compared to wild type, while ATP levels were higher.[131] These functional assays for Q indicate that neither Q levels, nor respiratory metabolism are greatly impaired in these worms. However, it is interesting to note that the *clk-1 e2519* mutant fails to mature in standard axenic medium.[131] This medium contains no *E. coli* as a food source and would lack a source of exogenous Q. The addition of autoclaved *E. coli* to the medium allowed for maturation. This could indicate that the worms may rely on *E. coli* (the standard food source for *C. elegans* in the laboratory) as a source of Q. The possible uptake of exogenously provided Q in the nematode system has not been studied, nor have assays that quantify Q directly in the *clk-1* mutants been performed. The extent to which the intriguing pleiotropic phenotype of *C. elegans clk-1* mutants depends on an exogenous source of Q merits careful investigation.

12.5 GENETIC EVIDENCE FOR A MULTISUBUNIT COMPLEX IN Q SYNTHESIS

There are multiple lines of evidence suggesting that a complex of enzymes may be involved in the synthesis of coenzyme Q. Six complementation groups of the *coq* mutants (*coq3–coq8*) all accumulate the same early intermediate in the Q biosynthetic pathway (**5**), suggesting that the absence of one *COQ* gene product prevents the function of the others.[71] One allele of *coq7*, a point mutation designated *coq7-1* (G_{104}-D) (Figure 12.6), accumulates intermediate **12**; however, as stated above, the null mutant accumulates only intermediate **5**.[123] Mitochondria isolated from yeast mutants containing an intact *COQ3* gene, but harboring deletions in any other *COQ* gene have decreased or absent levels of **8**:O-methyltransferase activity as compared to CoQ^+ respiratory defective control strains.[79] In fact, the Coq3 polypeptide levels were also greatly decreased in the family of *coq* null mutants, with the exceptions of *coq4* and *coq6*. Recent evidence indicates that the *COQ5* gene product, a *C*-methyltransferase, may have a secondary function in yeast, stabilizing Coq3p and its O-methyltransferase activity.[132] Five independent *coq5* mutant yeast strains have been characterized. Two of the three *coq5* mutants have amino acid substitutions that inactivate C-methyltransferase activity. These two mutants retain O-methyltransferase activity and are rescued by the expression of the *E. coli ubiE* gene. The three other *coq5* yeast mutants lack the *COQ3* encoded O-methyltransferase activity and cannot be rescued by expression of the *E. coli ubiE* gene. Taken together, these findings provide indirect genetic evidence that a complex of enzymes may be responsible for Q biosynthesis. There are precedents for this type of scheme, for example, the eukaryotic multisubunit respiratory complexes. In these systems, one missing or mutant component results in a drastic phenotype in which many related components are missing, unstable, or inactive.[133,134] This scheme would provide an explanation for the presence of **5** as the sole detectable Q-intermediate in the *coq3–coq8* null mutants. However, this observation is also consistent with coordinate regulation of the *COQ* genes. It is notable that this phenomenon is not observed in the *E. coli ubi* mutants, which tend to accumulate large amounts of each distinct Q intermediate.[135]

12.6 Q-LESS PHENOTYPES

In addition to being respiratory defective, both *E. coli* and yeast strains lacking Q display a wide range of phenotypes that reflect not only their energy-production defects, but their antioxidant status as well (for a recent review on the "Q-less" *E. coli* phenotypes see Soballe and Poole[11]). All of the *coq* mutant strains tested (*coq2*, *coq3*, *coq5*, *coq6*, *coq7*) exhibit hypersensitivity to polyunsaturated fatty acid treatment, specifically linolenic acid.[71,136] This sensitivity is not a secondary effect of the inability to respire, since other respiratory defective mutants fail to display this effect. Polyunsaturated

fatty acids are extremely prone to autoxidation and break down into toxic products that damage membranes, DNA, and proteins;[137,138] there was a marked elevation in both lipid hydroperoxides and aldehyde breakdown products found in the *coq3* mutant studied. This hypersensitivity in *coq* mutants could be rescued with a variety of antioxidants. This would indicate that coenzyme Q plays a critical role in protecting eukaryotic cells from toxic products not only by maintaining respiratory function, but by directly acting as a lipid soluble antioxidant as well.

The Q-deficient *S. pombe dps* mutant is unable to grow on minimal media containing glucose, but the defect can be rescued by supplementing such media with cysteine, glutathione, or α-tocopherol.[50] The *dps* mutant strain was also more sensitive to oxidative stress imposed by H_2O_2 or Cu^{2+}, a result that suggests a role for Q as an antioxidant in *S. pombe*. It will be of interest to evaluate other classes of Q-deficient *S. pombe* mutants to determine whether the phenotype of sensitivity to oxidative stress is generally observed as a result of Q-deficiency.

Yeast plasma membranes contain an electron transfer chain that maintains ascorbate in its reduced form; *coq* mutants display a decreased ability to stabilize ascorbate as compared with wild-type or other respiratory deficient mutants.[85,86] Reduction of ascorbate free radicals is apparently dependent on two activities, one based on an electron transport chain in which Q is the main carrier, and one dependent on the iron-regulated ferric reductase complex. In higher eukaryotes, extracellular ascorbate stabilization is important in maintaining an optimal redox state and may be related to effects on cell growth and differentiation.[139] For a discussion of plasma membrane Q, see Chapter 5 by Villalba et al., Extramitochondrial Functions of Coenzyme Q.

12.7 PERSPECTIVES

Q is widely used as a dietary supplement and in a variety of clinical therapies (the nutritional aspects of Q and the use of Q in clinical therapies are topics of discussion in later chapters of this volume). In most studies with young rats, dietary Q results in increased Q levels in blood (including lipoproteins) and liver, but has little effect on Q levels in other tissues.[140] Recently however, supplementation with Q in aged rats has been reported to improve age-associated arterial dysfunction,[141] stress tolerance and baseline heart function,[142] and to provide neuroprotective effects that correlated with significant increases in Q content in brain mitochondria.[143]

Q/QH_2 is the only lipid soluble antioxidant that can be synthesized by mammalian cells; the other lipid soluble antioxidants (vitamin E, β-carotene) must be derived from the diet. The studies reviewed in this chapter employed a combination of genetics and biochemistry to delineate the biosynthetic steps responsible for production of Q. Just as an understanding of cholesterol synthesis and metabolism provided important insights for control of LDL cholesterol levels in patients with hypercholesterolemia,[38] it seems likely that characterization of the biosynthesis of Q will benefit our understanding of Q metabolism, its possible role in aging, and the use of Q in clinical therapies.

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13 Dietary Intake and Absorption of Coenzyme Q

Christine Weber

CONTENTS

13.1 INTRODUCTION

Despite a large number of studies regarding the potential beneficial effects of CoQ_{10} supplementation, relatively little is known about the importance of dietary CoQ_{10}. The following summarizes the present knowledge of the content of CoQ_{10} in foods, the total dietary intake, the extent of intestinal absorption, and finally presents an evaluation of the significance of dietary CoQ_{10}.

Previous studies have suggested that reduced CoQ_{10} exerts an antioxidative effect in LDL particles,[1–3] and therefore the concentration and redox status of CoQ_{10} in the vascular compartment might be an important parameter for protection against atherosclerosis. In addition, plasma CoQ serves as a potential source of tissue CoQ. Plasma CoQ is present at a relatively high concentrations in healthy individuals (approximately 1 μM), a level that can be significantly increased by oral administration of CoQ_{10}, potentially enhancing the antioxidative capacity of plasma lipoproteins and contributing to the tissue concentration.

Dietary supplementation with CoQ_{10} has become increasingly popular in the Scandinavian populations, including the Danes, where 5.6% of the adult population in 1996 reported that they were currently taking dietary CoQ_{10} supplements,[4] although neither recommendations regarding CoQ_{10} intake, nor any final evidence for the beneficial effects of these supplements exists in healthy subjects. The strategy for dietary supplementation is to ensure that the individual receives the RDA of the micronutrients. Currently the optimal intake of CoQ_{10} is unknown. To evaluate the dose for CoQ_{10} supplements, it is interesting to compare the amount of CoQ_{10} in supplements to the amount naturally present in the daily diet.

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13.2 CONTENT OF CoQ IN THE DIET

Different foods contain a number of different CoQ homologues, of which CoQ_{10} and CoQ_9 are most prominent. The following discussion will focus on CoQ_{10}, as it represents the physiologically most relevant CoQ homologue in man.

13.2.1 CoQ in Dietary Items

Most dietary items contain CoQ due to the ubiquitous presence of mitochondria. However, many foods (such as a number of different vegetables and cereals) contain primarily CoQ_9, and some foods contain only traces of CoQ_{10}. The dietary items containing measurable amounts of CoQ_{10} can be divided into rich sources (above 20 μg/g, Figure 13.1) and less rich sources (below 20 μg/g, Figure 13.2). The CoQ_{10} rich foods (Figure 13.1) are mainly meat (8 to 200 μg/g), poultry (17 to 21 μg/g), and fish (4 to 64 μg/g) in accordance with a high content of mitochondria in muscle tissue. Other rich sources are certain vegetable oils (soybean, rapeseed, sesame) and nuts.[5] Fruit (0 to 5 μg/g), vegetables (0 to 10 μg/g), egg (2 to 4 μg/g), dairy products (0 to 2 μg/g), and cereals (containing primarily CoQ_9) are all moderate sources of CoQ_{10} (Figure 13.2). In contrast to soybean and rapeseed oil, other dietary fats, such as olive oil (4 μg/g) and corn oil (13 μg/g), although rich in CoQ_9) are less prominent CoQ_{10} sources, and coconut oil contains no detectable CoQ at all.[5]

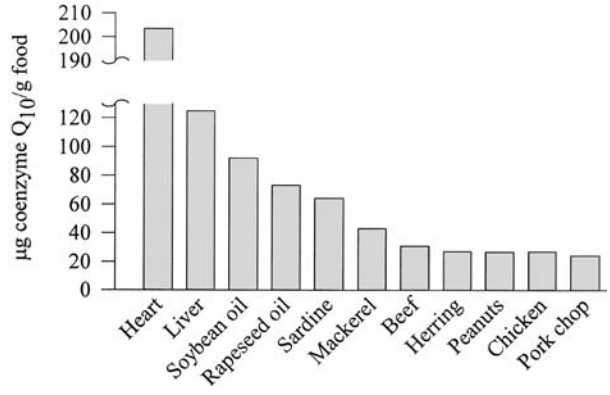

FIGURE 13.1 Dietary items with a high content of CoQ_{10}.[5,6,26]

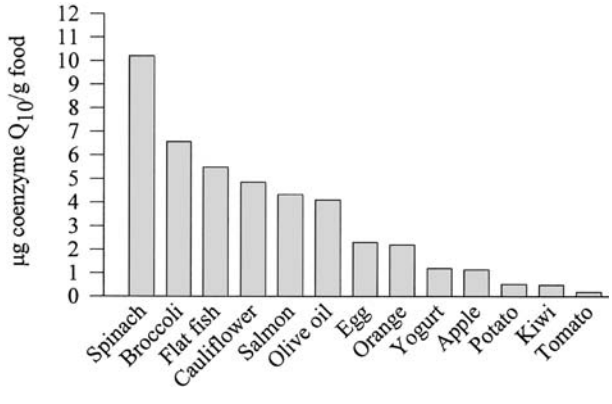

FIGURE 13.2 Dietary items with a moderate content of CoQ_{10}.[5,6]

TABLE 13.1
The Daily Contribution of Different Food Groups to the Dietary Intake of CoQ_{10} in the Danish Population[6]

Food Group	Contribution to CoQ_{10} Intake %	Intake g/day	CoQ_{10} mg/day
Cereals	0	227	0–0.03
Egg	2	36	0.06–0.08
Fruit	5	154	0.2
Poultry	5	13	0.2
Dietary fats	6	79	0.2–0.3
Dairy products	7	426	0.1–0.5
Vegetables	7	270	0.3
Fish	9	26	0.4
Meat	59	107	2.0–3.0
Total	100	1338	3–5

13.2.2 Effect of Cooking

As dietary CoQ_{10} is potentially labile during heating, the effect of cooking was examined.[6] Frying of meat (pork heart, pork chop) caused a loss of CoQ_{10} in the range of 14 to 32%, whereas CoQ_{10} in vegetables and eggs was unchanged upon boiling. This indicates a relatively low degree of CoQ_{10}-destruction during heat treatment of food items, and the data for raw foods may be considered representative for a cooked meal.

However, CoQ_{10} rich dietary fats are presumably less rich sources when used for frying, due to destruction during heating, which is known to cause major (up to 80%) destruction of vitamin E.[7]

13.2.3 Estimated Daily Dietary CoQ_{10} Intake

In order to evaluate the role of the diet as a source of plasma or tissue CoQ_{10}, it is mandatory to know the total amount of CoQ_{10} provided by the diet. The contribution of different food groups to the total CoQ_{10} amount illustrates whether any special dietary habits would have an effect on the CoQ_{10} intake.

Using food consumption data for the Danish population[7] and data for CoQ_{10} in Danish dietary items,[6] the average intake of coenzyme Q_{10} was estimated to be 3 to 5 mg/day (Table 13.1). The major contributions arose from the intake of meat and poultry, representing 64% of the daily CoQ_{10}. Cereals, vegetables, and fruit only made minor contributions, and fish, although relatively rich in CoQ_{10} (4 to 27 μg/g), only provides 9% of the total intake, due to the low consumption. Edible fats are only a minor source of CoQ_{10} (6%), since many of these primarily contain CoQ_9.[5]

Substantially different intakes of the rich CoQ_{10} sources (such as meat, poultry, and fish) will influence the intake. The Danish mean intake (1985) was 120 g meat and poultry per day, ranging from 43 g (5% fractile) to 245 g (95% fractile).[7] This would reflect a change in the estimate from the present 3 to 5 mg/day (intake of all other food groups presumed constant) to 2 to 3 mg in the low range and 6 to 9 mg in the high range. A Danish National survey from 1995 did not reflect any changes in the average CoQ_{10} intake, due to a relatively constant intake of meat and poultry.[8] The Danish diet is presumably representative for Northern Europe, but even with different dietary habits it is likely that most individuals have a dietary intake below 10 mg CoQ_{10}/day.

13.3 INTESTINAL ABSORPTION

In order for the dietary CoQ_{10} to have any physiological impact in man, it must be absorbed to a significant degree. Studies of the metabolism of CoQ_{10} in humans are complicated by the fact that the component is

endogenously synthesized. This could be overcome by studying CoQ_{10} metabolism using isotope-labeled CoQ_{10}. So far, the efficacy of absorption using radioactive tracers has been studied in rodents, but human studies directly investigating the extent of intestinal absorption of CoQ_{10} have not yet been published.

13.3.1 Route of Absorption

In the rat, CoQ_{10} is incorporated into chylomicrons after intestinal absorption, transported via the lymphatic system to the circulation,[9] and incorporated into VLDL particles in the liver.[10] In humans, hexahydroubiquinone-4, a CoQ_4 analogue used as a model component for CoQ_{10}, was recovered from the lymph after oral administration.[11] After oral administration of 200 mg CoQ_{10} to healthy volunteers, the majority was recovered after 6 hours in the triacylglycerol-rich lipoprotein fraction (VLDL and chylomicrons), but also detected in all other lipoprotein fractions.[1] Thus, it is likely that the absorbed CoQ_{10} is incorporated into lymph chylomicrons, released to the circulation, eventually taken up by the liver, and reincorporated into VLDL particles. Exchange reactions between lipoprotein particles are likely to occur at all stages of the absorption, as is the case for vitamin E, thus rendering absorbed CoQ accessible to all lipoprotein fractions.[12]

13.3.2 Efficacy of Absorption

Studies in rodents reveal an efficacy of absorption of CoQ from 1 to 8% of the dose.[9,13–16] It appears as if increasing dose, as well as the formulation has an effect, and that the unsaturation of the CoQ side-chain is important for efficient absorption.[13] Some studies use CoQ_{10} as a tracer in rats, which is problematic as rats have CoQ_9 as the major homologue and discrimination towards the endogenous homologue during absorption may exist. The factor also complicates the interpretation of CoQ_4-analogues as a model for CoQ_{10} in humans.[11]

In humans, the pharmacokinetic behavior of CoQ_{10} has been studied.[17,18] The plasma concentration has a maximum after approximately 6 hours. The amount of absorbed CoQ_{10} present in plasma at the time of the peak can be calculated, assuming a 2.5 L total plasma volume (Table 13.2). This amount corresponds to a minimal absorption, and the total absorption is presumably somewhat larger. It is seen that the absorbed amount tends to decrease with increasing dose, as is the case for vitamin E.[19] The total absorption is likely to be less than 10%, which is low, but consistent with the data obtained from the animal experiments.

13.3.3 Intestinal Absorption of Dietary CoQ_{10}

CoQ_{10} is known to be absorbed from supplements, as plasma CoQ_{10} increased significantly (51 to 164% above baseline levels) in subjects after repeated supplementation with relatively high doses (90 to 150 mg/day).[20–24]

TABLE 13.2
The Amount of CoQ_{10} Present Plasma Following a Single Oral Dose at the Time of the Peak Plasma Level, Assuming a 2.5 L Plasma Volume

CoQ_{10} Dose mg	*n*	Start CoQ_{10} mg/L	Peak CoQ_{10} mg/L	Absorbed at Peak % if Dose	Reference
30	9	0.97	1.44	4.0	25
100[a]	16	0	0.95	2.4	17
333	8	1.42	3.82	1.8	18

[a] Deuterated CoQ_{10} was used.

TABLE 13.3
Plasma CoQ_{10} Concentrations (mg/L, mean ± SD) of 9 Subjects Following Ingestion of 30 mg Coenzyme Q_{10}, Either as Capsules or Contained in a Meal

Time [h]	0	1	2	3	4	5	6	7	8	9
Capsule	0.88 ± 0.19	0.92 ± 0.19	0.91 ± 0.20	0.88 ± 0.18	0.92 ± 0.20	1.06 ± 0.25[a]	1.19 ± 0.36[a]	1.11 ± 0.37[a]	1.04 ± 0.29[a]	1.03 ± 0.31
Meal	0.97 ± 0.19	0.95 ± 0.19	0.99 ± 0.20	1.04 ± 0.23	1.21 ± 0.30[a]	1.22 ± 0.28[a]	1.44 ± 0.36[a]	1.31 ± 0.31[a]	1.21 ± 0.30[a]	1.30 ± 0.26[a]
Control	0.86 ± 0.21	0.89 ± 0.24	0.89 ± 0.27	0.91 ± 0.26	0.87 ± 0.23	0.88 ± 0.25	0.88 ± 0.21	0.91 ± 0.22	0.89 ± 0.23	0.84 ± 0.22

[a] Significant increases ($p < 0.01$) above baseline ($t = 0$ h). The control values show the plasma CoQ_{10} concentrations (mean ± SD) for 3 of the 9 subjects following ingestion of a meal with low CoQ_{10} content.[25]

As food items contain CoQ_{10} in a more complex matrix (embedded in membranes) than capsules (dispersed in soybean oil), it could be anticipated that the bioavailability of CoQ_{10} from the diet would differ from that of supplements. This was investigated by measuring the absorption of CoQ_{10} administered either as a meal or as capsules in healthy volunteers.[25] Pork heart was chosen for the test meal due to its high content of CoQ_{10}, and because heart tissue resembles other types of meat, which deliver the majority of dietary CoQ_{10}. After ingestion of a meal with low CoQ_{10} content, the plasma concentration was constant over time (Table 13.3, control). Therefore, the increase following ingestion of CoQ_{10} could be interpreted as absorption of CoQ_{10}. Capsules containing 30 mg CoQ_{10} resulted in significantly increased concentrations from 5 to 8 hours, and a meal containing 30 mg CoQ_{10} resulted in significantly increased concentrations from 4 to 9 hours (Table 13.3). The maximum concentration reached after 6 hours did not differ significantly between the CoQ_{10} sources. This demonstrates that CoQ_{10} can be absorbed from a meal. If CoQ_{10} in other types of meat are absorbed in a similar manner, it may be concluded that the diet is an important source of CoQ_{10} for the circulation.

13.4 SIGNIFICANCE OF DIETARY CoQ

13.4.1 Contribution of Dietary CoQ_{10} to Plasma Levels

In the rat, CoQ is known to be synthesized de novo in the liver and incorporated into VLDL particles, thus providing CoQ for the circulation.[10] In man, the ratio between de novo synthesis and absorption from diet on plasma CoQ_{10} levels is presently unknown.

Although the CoQ_{10} absorption from the diet is low, the dietary CoQ_{10} is likely to be important for the plasma CoQ_{10} concentration. Assuming that the mean daily CoQ_{10} intake is 5 mg, and that 10% of the ingested dose is absorbed, the daily contribution of the diet to the plasma level is 500 μg. As the circulating amount of CoQ_{10} is approximately 2000 μg, the daily dietary amount contributes 25% of the plasma CoQ_{10}-pool (assuming a 2.5 L plasma pool, and an 800 μg/L plasma CoQ_{10} concentration).

If the elimination of CoQ_{10} is approximated by simple first order kinetics, the dietary CoQ_{10} can account for the observed level in plasma (800 μg/L), as the steady state level is around 920 μg/L (assuming a dietary intake of 500 μg/day, no endogenous synthesis, and a plasma CoQ_{10} half-life of 33 h[17,18]). There is reason to believe that the elimination is far more complicated than this, but the figure indicates that dietary CoQ_{10}, despite the low absorption, contributes significantly to the plasma concentration.

13.4.2 Supplements vs. Diet

Currently, the dose of CoQ_{10} used therapeutically is in the range of 100 to 400 mg/day, which is far beyond the amounts that can be obtained from dietary intake. The optimal daily dietary intake for healthy individuals is unknown.

When considering the intake of CoQ_{10}, a clear distinction between the therapeutic use and supplementary use (to make up for a poor diet) should be drawn. CoQ_{10} supplements are sold in formulations of 10 to 100 mg/day, and typically recommended at doses of 30 to 60 mg/day, as judged by Internet advertisements (June 1999). This typical dose is not aimed at any of these strategies, as it is too high for the supplemental and too low for the therapeutic use.

13.5 CONCLUSION

Dietary CoQ_{10} is present in significant amounts (approximately 5 mg/day in the Danish diet). The degree of absorption is small, probably in the range of 5 to 10% of the intake. Still, dietary CoQ_{10} can contribute to the plasma pool, thus providing a potential antioxidative protection for plasma lipoproteins and a source for CoQ_{10} enrichment of peripheral tissues. It might be a significant factor in conditions with impaired plasma or tissue CoQ_{10} levels. In light of the large number of studies regarding the potential beneficial effects of CoQ_{10} supplements, further studies on the factors governing absorption, delivery to tissues, extent of endogenous synthesis, and excretion in man remain to be established.

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Section 2B

Dietary Supplementation and Plasma/Tissue Concentrations

14 Protection Against Oxidative Stress by Chronic Administration of Coenzyme Q

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CONTENTS

14.1 INTRODUCTION

The bioenergetic role of coenzyme Q (CoQ) in mitochondria as well as its essential role in other redox chains is well accepted. What is more often debated is its antioxidant function and the rationale leading to clinical administration. In spite of the suggestion that CoQ in mitochondria may be involved in oxygen free radical generation,[1] evidence has been found that CoQ acts as an antioxidant both *in vitro*[1,2,3,4,4a] and *in vivo*.[5] Thus, the reasons justifying the use of CoQ in different clinical conditions may arise both from improvement of cellular bioenergetics and antioxidant protection[3,6] and we shall try to critically discuss whether its therapeutic effect can reasonably be ascribed to the former or the latter of its features.

14.2 COENZYME Q DEFICIENCY IN TISSUES AND PLASMA

The first studies evidencing a certain degree of CoQ deficiency in myocardial tissue date to the first half of the 1970s[7,8] and show that 75% of patients undergoing cardiac surgery were affected by this deficiency. This finding was obtained through the enzymatic assay of succinate dehydrogenase-CoQ reductase of mitochondria prepared by intraoperatory biopsies. Thanks to the same technique, similar results were concomitantly obtained in the heart muscle of rabbits fed with a vitamin E deficient diet,[9] in the heart muscle of mice affected by hereditary muscle dystrophy,[10] in human gingiva of subjects affected by periodontal disease,[11] and in human muscle of patients affected by muscular dystrophy.[12] Later in 1984, it was found, through HPLC analysis conducted on endomyocardial biopsies, that patients in NYHA classes III and IV had lower cardiac CoQ concentration

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if compared to patients in classes I and II.[13,14] So, apparently, a certain degree of CoQ deficiency would occur at least in specific diseases and such deficiency might reasonably constitute the rationale for the therapeutic use of CoQ. The reasons for these lowered levels remain unclear; either a hypothetical impairment of CoQ biosynthesis, or an accelerated catabolism of CoQ are likely. Nevertheless, a possible explanation could reside in an augmented "antioxidant engagement" of CoQ, induced by changes in the tissue's homeostasis that somehow lead to its increased consumption and finally to its deficiency. This result may have dramatic consequences on the effectiveness of the mitochondrial redox chain. At this level CoQ acts as a substrate-like molecule, its diffusion is not rate-limiting for electron transfer, and its concentration is usually tenfold the other redox components, but it does not saturate the respiratory chain.[15,16,17] In other words, CoQ concentration in mitochondrial respiratory chain is in the range of the K_m for the enzymes that use it. An exogenous administration of CoQ is therefore hypothetically able to increase the velocity of electron transfer, particularly in all those cases of diminished CoQ content (due to metabolic reasons and/or accelerated levels of peroxidation). If CoQ is not saturating for the enzymes interacting with the CoQ pool, the overall enzymatic rate is a function of maximal velocity of the CoQ-dependent enzymes as well as of the total amount of CoQ.[16,17] Thus, even though CoQ concentration in the membrane is rather high, the velocity of the respiratory chain will strongly depend on the CoQ concentration and relatively small variations of the amounts of CoQ in the membrane will generate detectable changes in the respiratory rate. It follows that any process that leads to a certain decrease in the mitochondrial concentration of total CoQ or to excessively oxidizing or reducing conditions in the mitochondrial CoQ pool, resulting in a severe modification of the $CoQH_2$/CoQ ratio and consequent decrease of the concentration of either CoQ or $CoQH_2$, may be expected to inhibit mitochondrial proton movement and ADP phosphorylation and to produce serious physiological and medical problems. This last concept helps us to understand the rationale that links together the classical bioenergetic, proton-activating role of CoQ and its antioxidant role. Any condition of increased oxidative stress leading to enhanced involvement of CoQ as an antioxidant might somehow decrease the critical availability of CoQ itself for oxidative phosphorylation. On the other hand, it also helps to understand a possible mechanism of action of exogenously supplied CoQ, which, by overcoming a deficiency, could reestablish a higher energy flow and an enhanced energy transduction.

In line with these considerations, it is interesting to note that different bioenergetic or antioxidant roles for each of the two CoQ homologues present in animals like rodents has been suggested.[18]

Another mechanism was postulated[19] indicating that exogenously administered CoQ could have therapeutic effects as part of the redox-therapy strategy. According to this hypothesis, better availability of CoQ_{10} as a result of its exogenous supplementation, may enhance NADH oxidation at the mitochondrial level, and also at the cytoplasmic level, where it would act as a true "redox sink," i.e., as an electron acceptor for the plasma membrane-associated NADH dehydrogenase. As Linnane postulates "in the aging [sic] process natural, endogenous redox sinks other than the pyruvate/lactate couple would play an important role in enabling the individual to maintain an adequate bioenergy capacity in the face of declining mitochondrial oxidative phosphorylation function."[19]

Aging,[20] cardiovascular diseases,[21] dietary habits,[22,23] physical exercise and training,[22,23] and pharmacological treatments,[24] affect CoQ contents and alterations can vary significantly, depending on the tissue considered. Treatment with potent hypocholesterolemic agents, inhibitors of HMGCoA reductase, is also known to affect blood and tissue levels of CoQ_{10}, since biosynthesis of the isoprenoid chain is, up to a certain point, common to cholesterol and dolichol biosynthesis.[25,26,27,28]

CoQ blood levels, again, are affected by multiple situations even though it is likely they are inversely correlated with metabolic demand.[29] A further cause of low plasma CoQ is related to the increased level of peroxidation, at tissue level, arising from the ischemia-reperfusion damage[30] or from physical training.[23] The common feature of all these different situations is that plasma CoQ could play a diagnostic role, by reflecting some biochemical events taking place at the tissue level.

14.3 COENZYME Q ADMINISTRATION AND PEROXIDATION PRODUCTS

Based on the antioxidant role of CoQ, several approaches have been followed in trying to correlate CoQ administration and the extent of peroxidative damage.

CoQ_{10} is the homologue available for therapeutic administration and also, in some liposomal preparations, for *in vitro* experiments. Therefore from now on, we will use the generic expression CoQ to mean coenzyme Q at large, which could be CoQ_{10} or a shorter homologue present at significant levels in tissues other than human ones, or CoQ_{10} where this was specifically administered.

Involvement of oxidative stress in ischemia-reperfusion is well known and two kinds of studies have mainly been conducted for elucidating the role of CoQ in this syndrome. The first is an experimental model using the whole animal or the isolated, perfused heart, where ischemia-reperfusion is achieved through the temporary ligation of a coronary branch. The second approach requires the evaluation of biochemical and functional parameters in animals treated with CoQ before being subjected to the experimental procedure of ischemia-reperfusion. The results of experiments conducted, (using the electron spin resonance technique), on mitochondria isolated from rabbit heart subjected to a 40-min occlusion of coronary artery branch[31] indicated that mitochondria from ischemic heart generated higher levels of hydroxyl radicals and contained lower levels of CoQ. This finding was more pronounced when the mitochondria were prepared from an ischemic-reperfused heart. Later an experiment was conducted in a group of dogs that had been treated with CoQ_{10}, i.v., just before cardiac explantation.[32] The hearts were then stored in a preserving medium at 4°C for 6 to 24 h, before reperfusion. Myocardial levels of ATP were deeply affected by the storage time, i.e., by the length of the cold ischemia period. Pretreatment with CoQ_{10} consistently prevented the ATP decrease during ischemia and significantly shortened the recovery time for ATP after reperfusion. The effect of CoQ_{10} pretreatment was evident primarily in relation to oxidative damage. There was a great increase in the production of malondialdehyde (MDA) upon reperfusion, but only in the animals that received a placebo: in the CoQ_{10}-pretreated animals, elevation of MDA was negligible. In situations of experimental coronary ischemia and reperfusion, recovery of myocardial contractility is significantly faster in the CoQ_{10}-treated animals. Furthermore, pretreatment with CoQ_{10} also increases the ventricular fibrillation threshold, while minimizing the contractility impairment during experimental ischemia.[33]

Myocardial stunning, defined as a reversible decrease in contractility after ischemia and reperfusion, is a common clinical problem occurring after thrombolytic therapy or coronary bypass surgery. Even though its pathophysiological mechanism remains unknown, it is most likely a form of reperfusion injury. In order to check the possible effects of CoQ on stunning, Atar et al. used a normothermic open-chest model in the pig with short occlusion of the distal left descending coronary artery, followed by reperfusion.[34] The stunning time was significantly reduced in CoQ_{10}-pretreated animals as compared with placebo (13.7 ± 7.7 vs. 32.8 min). Moreover, plasma levels of reduced CoQ were higher in the CoQ_{10}-treated animals (0.45 vs. 0.11 μg/ml).

Myocardial preservation with CoQ_{10} therapy during heart surgery was also demonstrated in human patients.[35] Myocardial protective effect of CoQ_{10} was determined in high risk patients pretreated with CoQ_{10} for 15 days before and 30 days after heart surgery. After cardiac cooling, rewarming, and reperfusion, blood and tissue CoQ_{10} and tissue ATP levels were maintained in the normal range in CoQ_{10}-treated patients. Cardiac pumping and ejection fraction were significantly improved. The recovery course was short and uncomplicated, whereas it was long and complicated in the placebo group. Significant beneficial effect of CoQ_{10} on work capacity and a significantly lower level of plasma MDA in CoQ-treated patients compared to the placebo group was also demonstrated in a double-blind clinical trial that we conducted on postinfarctual ischemic patients.[36]

14.3.1 CoQ_{10} Administration and Oxidative Stress at the Myocardial Level

In a study conducted by our group in cooperation with a cardiology unit a few years ago,[37] we investigated whether protection against cardiac oxidative stress could be obtained by chronic CoQ_{10} supplementation. Wistar rats supplemented with CoQ_{10} (5 mg/kg/day) for 4 weeks showed a significantly higher level of CoQ in their left ventricle compared to controls. In the hearts perfused according to a modified Langerdorff technique, the oxidative stress was achieved by infusing H_2O_2 (60 μM) for 60 min. Marked alterations of both developed pressure, which decreased by 59 $\pm$ 17% and end-diastolic pressure, which increased almost 13-fold, were elicited in the control group. These effects were significantly reduced in hearts of CoQ_{10} supplemented rats ($-14 \pm 2\%$ and $+375 \pm 42\%$, respectively). Furthermore, cumulative release of oxidized glutathione from the hearts of the CoQ_{10} treated animals was one-fifth compared to the control group. In papillary muscles, also after 60 min. of perfusion with H_2O_2, active tension decreased to a large extent in controls whereas it was almost unchanged in the CoQ-treated group. Resting tension increased in papillary muscles of the control group about 30-fold compared to the CoQ-treated one. On the other hand, action potential duration and amplitude were shortened in the controls, who showed a progressive reduction. These effects could be interpreted as a specific consequence of oxidative injury, since inotropic and chronotropic responses to isoprotenerol were not different in control and CoQ_{10} treated rats.

Myocardial dysfunction associated with lipid peroxidation is known to occur after reoxygenation of hypoxemic hearts in the immature piglet subjected to cardiopulmonary bypass (CPB). Morita et al.,[38] tested the hypothesis that CoQ_{10}, administered before the onset of reoxygenation on cardiopulmonary bypass, can reduce oxygen-mediated myocardial injury and avoid myocardial dysfunction after CPB. It was found that CPB without hypoxemia caused no oxidative damage and allowed complete functional recovery. Hearts that were reoxygenated after hypoxemic CPB lasting 30 min showed a progressive increase in conjugated diene levels in coronary sinus blood after reoxygenation and a reduced antioxidant reserve capacity resulting in severe postbypass dysfunction. Conversely, CoQ_{10} treatment avoided the increase in conjugated diene levels, retained normal antioxidant reserve, and allowed nearly complete recovery of function. In this experiment, CoQ_{10} (45 mg/kg) was added to the cardiopulmonary bypass circuit 15 minutes before reoxygenation. Therefore, in this experimental model, CoQ_{10} administration was certainly acute, nonetheless reoxygenation of the hypoxemic heart on cardiopulmonary bypass causes oxygen-mediated myocardial injury, which can be limited by CoQ_{10} treatment before oxygenation. The effect of CoQ_{10} seems to be related, also in this case, both to its bioenergetic and antioxidant roles. Furthermore, recent data indicate that both endothelium-dependent and endothelium-independent arterial relaxation can be improved by increased dietary CoQ_{10} in aged rats.[39]

Recently, another study investigated the effects and relationships between the administration of different unsaturated dietary fats with and without the concomitant chronic administration of CoQ_{10} for 6 and 12 months on rat hearts.[40] The administration of CoQ_{10} was ineffective in changing any of the parameters concerning fatty acid composition of heart mitochondrial membrane and not even CoQ_9 and CoQ_{10} concentrations in heart mitochondria were affected. In this case the only remarkable effect obtained was a significant reduction of hydroperoxide content that was especially evident after 12 months.

Moreover, it has been demonstrated that acute CoQ_{10} administration protects the myocardium from reperfusion injury.[41] In fact, an acute administration of liposomal CoQ_{10} (10 mg/kg i.v. 30 min prior to 25 min of normothermic ischemia and 40 min of reperfusion injury on a Langendorff apparatus) improves recovery of function, aerobic efficiency, CK activity, and recovery of PCr and ATP after reperfusion. These data suggest that CoQ_{10} would increase myocardial tolerance to ischaemia/reperfusion, presumably through its antioxidant properties as well as by improving oxygen utilization and high energy phosphate production.

14.3.2 CoQ_{10} Effect on the Senescent Myocardium

Linnane and coworkers[42] explored the effect of CoQ_{10} administration on the tolerance of the senescent myocardium to aerobic and ischaemic stress. The study was conducted both in rats and human atrial tissue. In the former group, CoQ_{10} pretreatment with daily intraperitoneal injections of 4 mg/kg/day for 6 weeks improved the recovery of senescent rat hearts after aerobic stress to match the behavior of young hearts. In human heart tissue, they demonstrated that (i) CoQ_{10} content is lower in human atrial trabeculae from >70-years-old patients, (ii) trabeculae from older individuals (>70 years) have reduced recovery of developed force after simulated ischaemia compared to younger counterparts (<70 years) and finally (iii) this age-associated effect may be prevented in trabeculae pretreated *in vitro* with CoQ_{10}.

14.3.3 CoQ_{10} Treatment and Ischemic Brain Lesions

Subarachnoid hemorrhage (SAH) in humans is frequently complicated by the development of a "delayed" cerebral vasospasm, which may result in ischemic brain damage and irreversible neurological deficit(s); these phenomena may be related to free radical injury, since protective effects of antioxidants in both experimental and clinical vasospasms have been reported.[43] In a rabbit model of symptomatic vasospasm[44] it was found that CoQ_{10} administration (10 mg/kg per day for 6 days) could prevent the development of ischemic brain lesions. The experimental design consisted of ligation of both common carotid arteries: two weeks later the rabbits underwent an injection of autologous blood into the subarachnoid space, which was repeated after a two day interval. Histological examination of brain hemispheres revealed a number of pathological changes in the control group. Multiple well-delineated focal lesions adjacent to small blood vessels were found in the cortex, basal ganglia, and hippocampus. Numerous focal lesions of a more diffused appearance were also found in the corpus callosum. On a cellular level, these lesions were suggestive of degeneration or disappearance of neurons and of myelin disintegration. On the contrary, no lesions were found in the brain hemispheres of CoQ_{10}-treated animals. None of the CoQ_{10}-treated rabbits displayed a detectable neurological deficit at the end of the experiment, while in the control group, three out of ten rabbits died before the end of the experiment and the surviving ones showed different degrees of neurological deficit. Therefore CoQ_{10} treatment was able to prevent the development of both the neurological deficits and histologically detectable brain tissue damage.

14.3.4 Effect of Chronic CoQ_{10} Supplementation on Plasma Lipoprotein Peroxidation

The role of CoQ in preserving plasma lipoproteins (mainly LDL) from peroxidative insult has been widely investigated. The contribution of Stocker and coworkers in this field has been determinant[45] and is reported elsewhere in this volume. In our lab, we also conducted several studies on the distribution of CoQ_{10} among plasma lipoproteins.[46] In a specific case[47] we investigated the effect of chronic administration of CoQ_{10} on low density lipoprotein peroxidizability. LDL particles are heterogeneous and differ in size, density, and chemical composition. It is possible to isolate different LDL subfractions named LDL_1, LDL_2, and LDL_3 according to increases in their density. LDL_3, the most dense of the three populations, and the more abundant in patients affected by coronary heart disease (CHD), has also shown an increased susceptibility to oxidation.[48] We demonstrated that chronic administration of CoQ_{10} (100 mg/day *per os* for 30 days) was able to markedly elevate CoQ_{10} content in all three LDL subfractions investigated, particularly LDL_3, which had the lowest CoQ_{10} content before supplementation. Concomitantly with such enrichment, the LDL_3 subpopulation showed a drastic decrease of hydroperoxides (50% of those detected before CoQ_{10} supplementation) supporting the hypothesis that CoQ_{10} endowment in LDL subfractions affects their oxidizability.

It should be considered that the bioavailabity of CoQ is limited. Dallner and coworkers demonstrated[49] that no dietary CoQ is recovered in the heart or in the kidney, while CoQ concentration clearly increased in plasma and in liver where it was located mainly in lysosomes. This fact would account for the difficulties in obtaining CoQ enrichment in different tissues. A partial confirmation was obtained in a placebo-controlled double-blind study with marathon runners supplemented daily with 90 mg of CoQ plus 13.5 mg of α-tocopherol for three weeks before a marathon.[50] Pretreatment with CoQ was effective in reducing the susceptibility of the VLDL + LDL fraction to copper-induced oxidation but the supplementation had no effect on lipoprotein peroxidation or on the muscular damage induced by exhaustive exercise.

In a similar study conducted by Bargossi et al.,[51] a group of marathon runners and competitive walkers underwent a 40 day treatment with 100 mg CoQ_{10} per day. Before and at the end of the supplementation period the athletes were subjected to a test consisting of 40 km controlled training at 80 to 85% of their anaerobic threshold. The increase, (induced by this standardized test), in the plasma level of creatine kinase, lactate hydrogenase , and myoglobin, which are commonly regarded as markers of membrane leakage, was significantly lower after CoQ_{10} treatment.

Coenzyme Q_{10} has been gaining considerable attention over the past few years as an agent capable of influencing cellular bioenergetics and counteracting some of the consequences of free radical mediated insult. Further assessment of its protective antioxidant role will reasonably benefit from new, affordable techniques, able to evaluate the degree of oxidative stress in patients.

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15 Analysis of Coenzyme Q in Biological Samples

Guy Rousseau and Christine Des Rosiers

CONTENTS

15.1 INTRODUCTION

Over the past decade, the classical biological role of coenzyme Q or ubiquinone, as an obligatory component of the mitochondrial electron transport chain, has been broadened to include a protective role against oxidative stress.[1–3] However, reports of a prooxidant activity of ubiquinone,[4,5] and its link to pathological processes such as free radical production and mitochondrial permeability transition pore opening,[6,7] indicate that our understanding of the biological role of ubiquinone is still inadequate.

Of clinical relevance, deficiencies in ubiquinone are implicated in many pathologies. Furthermore, these deficiencies may be worsened by treatment with drugs such as the hypocholesterolemic HMG-CoA reductase inhibitors or statins,[8–10] and some antihypertensive drugs.[11] Though beneficial effects following ubiquinone administration were noted in patients with cardiomyopathies,[12] including ischemic heart diseases, muscular dystrophies, and neurogenic muscular atrophies,[13] the mechanisms of action have not been elucidated. In many instances an extravascular effect is proposed, such as inhibition of LDL oxidation.[14] In fact, the extent to which exogenously administered ubiquinone is incorporated in cellular membranes remains to be elucidated.

Thus, the elucidation of ubiquinone's role provides an impetus for the development of methods for the assay of ubiquinone in biological samples. Through the years, methods were refined to avoid nonspecific loss of ubiquinone due to autooxidation during sample processing and to include internal

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standards (IS) for precise quantitation. More recently, with evidence suggesting that the ratio [oxidized(Q_{ox})]/[reduced(Q_{red})] of tissue or plasma ubiquinone, rather than total ubiquinone, could be a more valuable index of the oxidative stress status,[15,16] methods were published for the one-step chromatographic assessment of this ratio.

The purpose of this review is to provide general guidelines for the selection of a method for the analysis of ubiquinone in biological samples. First, we briefly review the chemistry, biology, and tissue distribution of ubiquinone, which are relevant to its successful analysis. Then, we describe major guidelines gathered from a review of 39 methods published since 1978, emphasizing obligatory steps to be followed in the determination of tissue levels of ubiquinone. These include considerations of the choice of external or internal standards and conditions for storage, homogenization, organic extraction, chromatography, and detection. We then list criteria for selection and validation of the method likely to best suit each respective goal. Factors to be considered in the selection of these methods are numerous. Aside from simplicity, reproducibility, and detection limit, these factors include species, tissue of interest, age, hormonal and nutritional status, all of which are determinant for the tissue levels of ubiquinone and the nature of the predominant chemical form of ubiquinone, Q_{ox} and/or Q_{red}. In view of the abundance of the literature, we refer to review articles whenever available, otherwise we refer to the original article.

15.2 OVERVIEW OF BASIC KNOWLEDGE ON UBIQUINONE

The choice and validation of the methods for the study of ubiquinone biology requires an understanding of the chemical and physical properties of ubiquinone. Furthermore, the application of any given method to a given problem requires the knowledge of the tissue distribution and levels of ubiquinone as well as factors possibly influencing the tissue levels of ubiquinone. For further details, please refer to other sections of this book.

15.2.1 Chemical and Physical Properties of Ubiquinone

Ubiquinone is a lipophilic molecule, consisting of a quinone ring with an isoprenyl side chain (Figure 15.1). The size of the isoprenyl chain varies with species. For example, in humans, the predominant form of ubiquinone contains ten isoprene residues (Q_{10}), whereas in rats, the predominant form contains nine residues (Q_9). In tissue, ubiquinone is present in two different forms: the reduced form (Q_{red}), referred to as ubiquinol, and the oxidized form (Q_{ox}) or ubiquinone. For the remainder of this review, we will use the abbreviation Q_{ox} to refer to the oxidized form and Q_{red} for the reduced form. Ubiquinone will serve as a general term, without any reference to the state of oxidation.

It is Q_{red} that bears antioxidant properties, whereas there are reports that the Q_{ox} could act as a prooxidant.[4,5] Ubiquinone can also exist as the chemically unstable ubisemiquinone, which results

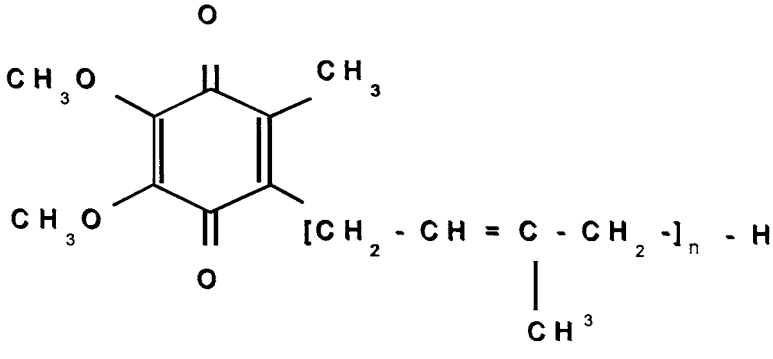

FIGURE 15.1 Chemical structure of ubiquinone (n represents the number of isoprene residues).

from the univalent reduction of Q_{ox}.[3] *In vivo*, the interconversion between Q_{ox} and Q_{red} is catalyzed by enzymatic processes, such as occur during cellular respiration. Q_{red} can also be converted to Q_{ox} in the process of radical quenching. *In vitro*, Q_{red} is prone to oxidation in alkaline ethanolic solutions, while it is stable at physiological pH values.[5]

15.2.2 Distribution and Levels of Ubiquinone in Tissue

Ubiquinone is present in all cellular membranes and in blood lipoproteins. Levels of ubiquinone are 0.5 to 2 μM in plasma,[17] and vary greatly in tissues, ranging from 0.5 to 300 nmol/g. Although the relative distribution of ubiquinone in tissues varies by species, the highest concentrations are found in liver, heart, muscle, kidney, and brain, irrespective of the chain length of the isoprene side chain.[2,18–20] At the subcellular level (liver), ubiquinone is mainly found in the inner mitochondrial membrane, where it is an important component of the respiratory chain, the Golgi vesicles, and the lysosomes (Table 15.1).[21]

The ratio of $[Q_{ox}]/[Q_{red}]$ also varies greatly among tissues and species (see Table 15.2). For example, Q_{ox} represents 80 to 90% of total ubiquinone in rat and mouse myocardium, while it represents only 10 to 25% of total in rat and human plasma. In rat and mouse liver, the reported levels of Q_{ox} vary over a greater range, from 30 to 70%.

TABLE 15.1
Distribution of Ubiquinone in Subcellular Fractions in Rat Liver

Subcellular Fraction	Coenzyme Q_9 (μg/mg protein)
Homogenate	0.79 ± 0.08
Golgi vesicles	2.62 ± 0.15
Lysosomes	1.86 ± 0.18
Mitochondria	1.40 ± 0.16
Inner mitochondrial membranes	1.86 ± 0.13
Microsomes	0.15 ± 0.02
Peroxisomes	0.29 ± 0.04
Plasma membranes	0.74 ± 0.07
Supernatant	0.02 ± 0.004

SOURCE: (Reprinted from Kalén et al., 1987 [21] with permission).

TABLE 15.2
Proportion of Oxidized Ubiquinone (Q_{ox}) in Various Tissues

Tissue	Species	Q_{ox}(%) of total)	References
Myocardium	rat, mice	80–90%	[26, 48, 51, 79, 80]
Muscle	rat	60–90%	[35, 80]
Liver	rat, mice	30–70%	[26, 48, 51, 67]
Kidney	rat, mice	60–80%	[26, 48, 51, 67]
Serum	rat	>90%	[51, 79]
Plasma	human, rat	10–25%	[34, 39, 79]

TABLE 15.3
Factors Influencing Total Tissue Levels of Ubiquinone

A. Decrease

Aging (human)[24]
Hormonal status: Hypo- and hyperthyroid states[81]
Nutritional status: Vitamin E deficiency[82]
Drug treatment: Statins, antihypertensive drugs[11]
Diseases: Oxidative stress, hypercholesterolemia,[83] AIDS,[84] ischemic diseases,[85,86] hepatocellular carcinoma,[87] inherited metabolic diseases (mitochondriocytopathies, mevalonic aciduria due to HMG-CoA reductase deficiency),[88] degenerative muscular diseases,[89] and rejection episodes after transplantation.[90,91]

B. Increase

Ubiquinone supplementation[25]
Physiological status: Aging (rat liver),[24] cold acclimation,[2] endurance training[92]
Hormonal status: Thyroid status; decrease or increase with hypo- vs. hyperthyroid states[81]
Drug treatment: Peroxisome proliferators,[78] catalase inhibitor (aminotriazole), squalene synthesis inhibitors[93]
Diseases: Neurodegenerative diseases such as Alzheimer's, prion sickness in mice, hyperplastic nodules in rat liver[94–96]

15.2.3 Factors Influencing Tissue Levels of Ubiquinone

Ubiquinone is synthesized from acetyl-CoA by the cholesterol pathway. Several factors, known to influence the tissue levels of ubiquinone are listed in Table 15.3. The major factor known to influence the ratio $[Q_{ox}]/[Q_{red}]$ in blood or tissue is the level of oxidative stress.[1,22] For this reason, this ratio has been proposed as an indicator of the level of oxidative stress.[15,16]

It should be noted that factors listed in Table 15.3 might differentially influence ubiquinone levels in tissues. For example, from adulthood to death, levels of ubiquinone in humans gradually decrease in the myocardium, red muscle, and kidney, while it remains relatively constant in the brain and increases in the liver[2,23,24] [ubiquinone half-lives in tissues vary from 50 to 120 hours[2]]. Furthermore, though there appears to be a consensus that ubiquinone administration can increase its blood[25] and liver[26,27] content, whether ubiquinone levels in other tissues can be increased is debated. Indeed, in many studies, oral administration, acute or prophylactic, resulted in only slight tissue absorption.[26–29] This contrasts with other antioxidants such as vitamin E.

As a whole, much remains to be learned about the influence of the various factors on the distribution of ubiquinone, both at the cellular and subcellular levels, especially in humans.

15.3 METHODOLOGICAL CONSIDERATIONS

In this section, we have emphasized general guidelines (schematized in Figure 15.2) for the analysis of ubiquinone in biological samples. First, general considerations that apply to the entire analytical procedure are presented. Then, rules for tissue sampling, homogenization, and storage, followed by those for organic extraction of ubiquinone are listed. Finally, chromatographic and detection methods are described. For detailed analytical procedures, please refer to the original publications summarized in the Appendix entitled Available Methods for the Assay of Ubiquinone.

15.3.1 General Considerations

Let us first examine three points, listed in order of priority, which in our opinion, should be considered prior to choosing a method for ubiquinone analysis.

First, the main problem with ubiquinone analysis remains the possible oxidation of Q_{red} during sample processing and analysis. As a general rule, to minimize this problem, the handling of samples should be done as rapidly as possible, with samples as well as working solutions such as extracting

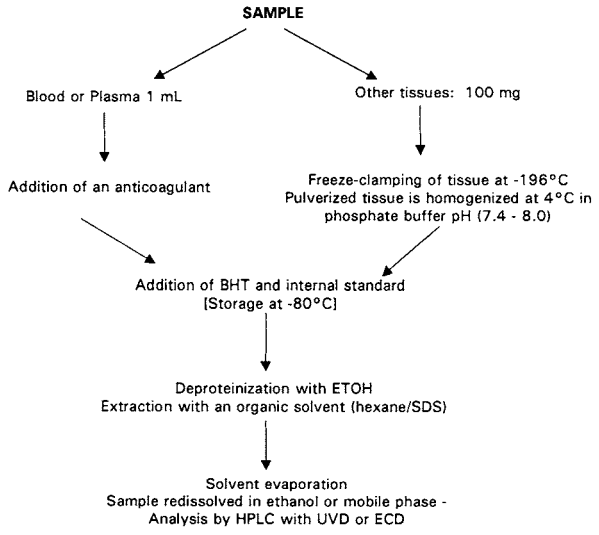

FIGURE 15.2 Overview of sample processing for ubiquinone analyses in tissue and blood.

solvent kept at low temperatures (4°C). Furthermore, the addition of an antioxidant should be considered.[30–32] This is most important for tissues such as plasma where ubiquinone is predominantly present in its Q_{red} form (> 90% of total). Powerful antioxidants should be avoided since they will reduce the oxidized form.[33] In the majority of studies, the antioxidant was butylated hydroxytoluene (BHT). BHT is completely soluble in all organic solvents and only a very small concentration (0.005%) is necessary to be effective.[32] The time of addition of BHT depends on the tissue analyzed, either at the time of sampling for blood, or at the time of homogenization for tissue. We noted that more recent methods for the determination of Q_{ox} in plasma have not used antioxidants. These methods have relied on rapid extraction methods since the oxidation of Q_{red} is minimized if samples are kept on ice and if the extraction lasts less than one hour.[34] Some authors also recommended photoprotection.[33,35] However, in our experience, photodegradation appeared to be minimal during sample processing.[36]

The second factor to consider in ubiquinone analysis is the approach for quantitation. Since sample processing for ubiquinone analysis includes steps such as organic extraction, the recovery of ubiquinone from one sample to another could vary and affect the reproducibility of measurements. Thus, consideration should be given to the addition of an internal or external standard. The quantity of standard added to the samples should be adjusted to correspond to the range (normally about 1.5 to 2 times greater on a molar basis) of the expected quantity of ubiquinone in these samples. The time of addition of this standard during sample processing varies among publications. For more precise quantitation, we recommend its addition at the earliest possible step, namely at the time of sampling for blood or plasma, or at the time of homogenization for tissues, and after subcellular fractionation whenever it applies. When the standard is added at an early step, it is referred to as internal standard, and will correct for any ubiquinone loss during the entire procedure. When the standard is added at a later step, such as following organic extraction, it is referred to as an external standard. An external standard does not correct for differences in ubiquinone recovery during the organic extraction. As for the choice of standard, we recommend the use of Q_{11}, or other ubiquinone analogues absent in the biological samples, since their physicochemical properties are similar to those of Q_9 and Q_{10} thereby minimizing the influence on the ubiquinone/internal standard ratios. Unfortunately, Q_{11} is not readily available (we obtained it courtesy of Nisshin Chemical Company, Tokyo, Japan). When the standard chosen is endogenously present, a technique referred to as standard addition, (simultaneous processing of identical samples in the absence or presence of a known quantity of this standard) has to be performed.[37] However, in one study[37] the recovery reported (64%) is lower than that reported with the use of an internal standard.

The third and last point to consider is sample size. The decision about sample size depends on the concentration of ubiquinone in the sample of interest and the sensitivity of the method chosen. In general, 1 mL of blood and 100 mg of tissue can be used to measure total ubiquinone levels. A greater amount of tissue, e.g., 1 to 5 g for liver,[21] is required for subcellular fractionation. Since, in many cases, the quantity of blood available for analysis is very small, it is noteworthy to report a method[38] that can be performed with only one drop of blood.

15.3.2 Sampling, Homogenization, and Storage

Blood and plasma samples, of about 1 mL, are frozen immediately after sampling. Either ethylenediaminetetraäcetic acid (EDTA) or heparin have been used[31] to prevent blood coagulation. Otherwise, the use of 5 mM ascorbate in 5 mM phosphate buffer pH 7.4 is recommended.[35] Tissue samples are rapidly freeze-clamped at the temperature of liquid nitrogen (−196°C), unless subcellular distribution of ubiquinone is sought. In the latter case, tissue is processed as rapidly as possible for subcellular fractionation with appropriate buffer at 4°C without freezing.[31] For the assay of total tissue ubiquinone, freeze-clamped tissue is pulverized at the temperature of liquid nitrogen and homogenized at 4°C, usually in a phosphate buffer pH 7.4. It is essential that the tissue be homogenized thoroughly as this greatly influences the efficiency of extraction of ubiquinone. We recommend adding BHT and the internal standard at this stage.

Samples can be stored either immediately after sampling or after tissue homogenization. Freeze-clamped tissues are stored at −196°C. Blood and plasma samples, and homogenized tissue extracts are stored at −80°C. During processing of tissue for subcellular fractionation or samples for extraction, the temperature is kept at 4°C. Plasma Q_{red} levels were reported to be stable at −80°C[39] for up to 13 months.[34] Repeated freeze-thawing of samples should be avoided, therefore we recommend freezing samples in small aliquots (1 mL for blood, 100 mg for tissues).

15.3.3 Organic Extraction

The procedure for processing of samples, either blood, plasma, or tissue homogenates is similar. It includes: (i) deproteinization with ethanol and (ii) extraction with an organic solvent. The combination and choice of organic solvents for extraction of ubiquinone influences its recovery from samples. The reported recovery of ubiquinone during organic extraction varies from 89 to 99%.[35,39]

Hexane and dichloromethane are the solvents of choice for the ubiquinone extraction, although some authors suggest the use of heptane because of its lower volatility.[40] In addition, the extractability of ubiquinone during *n*-hexane extraction is improved by the addition to the aqueous phase of methanol or ethanol at a concentration greater than 50%.[39, 41, 42] Furthermore, with tissue samples, the use of sodium dodecyl sulfate (SDS) results in a better separation of the aqueous and organic layers.[43] There appears to be an optimal concentration of SDS to obtain maximal efficiency of extraction, though reported optimal concentrations vary among authors, either 16 mM[43] or 100–200 mM.[44] Another advantage of SDS addition is cleaner HPLC traces (i.e., a lower background noise due to nonspecific contaminants).

15.3.4 Chromatographic and Detection Methods

Based on 39 publications screened for ubiquinone analysis, the separation is achieved with an HPLC equipped with a reverse phase type of column and an ultraviolet (UVD) or electrochemical (ECD) detector. Note that the chromatographic retention times for ubiquinone will vary with the choice of mobile phase solvents, as well as with its isoprene chain length and state of oxidation. Other additions to this basic system include a second column to achieve reduction or oxidation of ubiquinone. The latest and most sophisticated HPLC setup has the capacity to perform the one-step sequential analysis of tissue ubiquinone both as Q_{red} and Q_{ox}. The greatest difference between these various set ups is the detection limit for the analysis of Q_{red} and Q_{ox}. These differences can be summarized as follows.

TABLE 15.4
Criteria for Selection of Methods

Cost and availability of instruments
Simplicity
Reproducibility
Limit of detection—quantity of tissue available
Tissue of interest—determinant for the proportion of Q_{red} and Q_{ox}
Requirement for subcellular fractionation
Species—determinant for the nature of Q, for example Q_9 in rat vs. Q_{10} in human
Necessity to assay ubiquinone and another analyte simultaneously (e.g., another antioxidant such as vitamin E)
Experimental conditions tested: possible effect on the extent of oxidative stress

The UVD of ubiquinone has been achieved at 275 or 290 nm, which corresponds to the maximal peak absorbance for Q_{ox} and Q_{red}, respectively. However, the molar absorption coefficient of Q_{ox} at 275 nm (14,700 cm^{-1}) is greater than that for Q_{red} at 290 nm (4,100 cm^{-1}).[45] Consequently, the sensitivity for the analysis of Q_{red} with UVD is lower than for Q_{ox} and can become limiting for one's application. In contrast, with ECD, the detection limit is greater for Q_{red} than Q_{ox}.[46] To overcome these limitations, several strategies have been developed. For example, the two detection systems are combined and used in series, namely UVD for Q_{ox} and ECD for Q_{red}.[47–49] Others have used ECD detection alone,[15,46,50] but included a step for online reduction of Q_{ox} to Q_{red} using a chemically pretreated column[51,52] or a coulometric method[34,53] (i.e., with $NaBH_4$ or with platinum catalyst reduction postcolumn).[33,51] The detection limits obtained with various setups appear in the Appendix entitled Available Methods for the Assay of Ubiquinone.

15.4 SELECTION AND VALIDATION OF METHOD

15.4.1 Selection of Method

A list of the general criteria to be considered in the selection of a method for ubiquinone analysis is given in Table 15.4.

As a general rule, the measurements of both Q_{ox} and Q_{red} are initially recommended at least for validation purposes. However, once this is validated, we consider that UVD at 275 nm is the simplest and the lowest cost approach. Though it has its limitations, since it only detects Q_{ox} with great sensitivity, it can be used for the analysis of ubiquinone in tissue where Q_{ox} is the predominant form such as the myocardium and muscle (see Appendix). Other possible applications include the study of drug treatments, provided that pilot experiments documented no effect of the treatment on the ratio Q_{red}/Q_{ox}, for example, the effect of a treatment that affects the biosynthesis of ubiquinone, but not the enzymatic system responsible for its reduction, such as appears to be the case for the statins.[54–57]

However, in several studies[54–59] the tissue levels of ubiquinone as well as the Q_{ox}/Q_{red} ratio often changed considerably after treatment with drugs and chemicals. In these studies, as well as when analyzing tissues where the Q_{red} form is predominant, the measurement of both Q_{ox} and Q_{red} using one of the strategies described above becomes imperative. The greatest limitation for the choice of the strategy will probably be the availability of the setup. In this regard, one possible strategy to maximize the sensitivity of the assay with UVD method is to assay (i) Q_{ox} at 275 nm, and (ii) total ubiquinone ($Q_{red} + Q_{ox}$) as Q_{red} at 290 nm, after the chemical reduction of Q_{ox} to Q_{red} with $NaBH_4$. However, it must be remembered that the detection limit for Q_{red} at 290 nm is lower than that of Q_{ox}.

15.4.2 Validation of Method

Method validation should be performed to prove its suitability for the intended application. A revision of the general criteria for method validation is beyond the subject of this review, but has been the subject of publications.[60]

In summary, to demonstrate the suitability of the selected method, the following information should be provided.
(i) Standard curves in the tissue(s) of interest should demonstrate linearity in the appropriate concentration range and with the quantity of tissue. This is done by adding a fixed amount of an internal standard to (a) a fixed amount of tissues containing endogenous ubiquinone without or with variable quantities of exogenous ubiquinone, or (b) variable quantities of tissue with endogenous ubiquinone without or with variable quantities of exogenous ubiquinone. Ideally, the internal standard of choice should be added in concentrations similar to the peak of interest. It should elute close to the peak of interest, but must be completely resolved from any peaks in the sample. Data from this set of experiments will provide information on the precision, ruggedness, and selectivity of the method. It should allow for the detection of any contaminating peak. Additional tests to demonstrate the absence of a contaminating peak include subjecting the samples to conditions suspected to generate degradation products.
(ii) The reproducibility of replicate analyses of ubiquinone in the tissue(s) of interest should be lower than 5%. This is calculated by the formula of the coefficient of variations: %SD/mean. The intraassay precision and the interassay reproducibility can be tested by a number (*n*) of measures on one day, and by repeating *n* measures everyday for a number of days, at different volumes of injections.
(iii) Recovery of ubiquinone is best tested by directly comparing reponses of replicates of extracted samples with replicates of extracted blank matrix to which analyte has been added at the same nominal concentration.[60] In general, a recovery greater than 95% for samples with no internal standard should be obtained. Variable and lower recovery can be corrected with the use of an internal standard.

15.5 CONCLUSION

From the survey of the published methods on ubiquinone analysis, we conclude that there is a general agreement on the optimal conditions for sample storage, tissue homogenization, organic extraction, and chromatographic and detection methods. The use of only one detection method, either UVD or ECD, has potential limitations with respect to the detection limit for Q_{red} and Q_{ox}, respectively. The use of the more sophisticated setup for the simultaneous one-step analysis of Q_{ox} and Q_{red} has a great analytical advantage, though the availability, the complexity, and the cost of the instrumentation could be a limiting factor. In general, care should be taken in the method selection as not all published methods have been fully validated. Method validation is necessary for obtaining reliable data. As a whole, no sufficiently versatile method that could be applied with the same sensitivity and reliability to different biological samples, such as cultured cells, animal tissues, and biological fluids has been described so far. The main problem remains the possible oxidation of Q_{red} in the sample preparation and analysis. To minimize this effect, the following precautions should be taken into consideration: (i) the use of an excess of BHT (or another similar antioxidant); (ii) samples should be kept at low temperatures; and (iii) the handling of samples should be done as rapidly as possible. This is likely to be most difficult to achieve with subcellular fractionation of tissue.

In conclusion, a careful application of the published methods, which ought to include a crucial validation step, is to be considered to provide some answers to the unresolved questions regarding the biology of ubiquinone. These include: (i) how is ubiquinone synthesis and degradation regulated *in vivo*, (ii) how do conditions listed in Table 15.3, especially ubiquinone supplementation, differentially influence the levels of ubiquinone in the various tissues or at the subcellular levels, and (iii) what is the physiological significance of the variations in the ratio Q_{ox}/Q_{red} in various tissues?

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15.6 APPENDIX—AVAILABLE METHODS FOR THE ASSAY OF UBIQUINONE

Note: The methods have been grouped according to the tissues analyzed. A section on subcellular fractionation is also included. When more than one tissue is analyzed by a specific method, the method is listed within one single category according to the following priority: myocardium, muscle, liver, blood, and plasma with the other tissues mentioned in parentheses. In each section, the methods are listed by species and in chronological order of publication.

SPECIES (Other tissues-species)	HOMOGENIZATION/ EXTRACTION	HPLC ANALYSIS AND[OTHER COMPONENTS ANALYZED SIMULTANEOUSLY]	COLUMN SPECIFICATIONS	INTERNAL STANDARD (External standard/standard addition) LIMIT OF DETECTION AND [RECOVERY]	REFERENCE
		A. MYOCARDIUM			
human, rat (blood, liver, kidney, lung, brain)	- homogenization with H_2O - extraction with hexane/ETOH - redissolution in ETOH	- Q_{ox}/Q_{red}: ECD - ϕM: sodium perchlorate/ METOH/ETOH - [tocopherol]	C8, 5 μm 150 × 4.6 mm	menaquinone-8 100 pg [96%]	Wakabayashi et al., 1994 [51]
human (plasma)	- homogenization with propanol (photoprotection) - addition of $NaBH_4$	- Q_{ox}/Q_{red}: ECD -ϕM: propanol/METOH - on-line oxidation with ferric chloride - [tocopherol, cholesterol]	ODS, C18, 5 µm Glass 100 ×3 mm	Q_{10} (diethoxy analogue) 50 ng [88%]	Edlund, 1988 [33]
rat (liver, brain)	- extraction with propanol/hexane - redissolution in ETOH/H_2O_2	- UVD λ = 275 and 290 nm ϕM: METOH/ETOH	RP LC-18-DB, 5 μm 300 × 40 mm	none 1 ng [90%]	Graves et al., 1999 [61]
rat (muscle, blood)	- homogenization in H_2O/BHT/SDS - (ascorbate added for blood) - extraction with hexane/ETOH - redissolution in METOH/ETOH	- UVD: λ = 275 nm - ϕM: METOH/ETOH	ODS, C18, 5 μm 125 × 4.6 mm	Q_{11} 62.5 ng/mL [90%]	Rousseau and Varin, 1998 [36]
mouse (liver, kidney, brain, skin)	- homogenization in phosphate buffer/BHT/SDS - (ascorbate added to blood) - extraction with hexane/ETOH - redissolution in METOH/ETOH	- Q_{ox}/Q_{red}: ECD and UVD λ = 275 nm - ϕM: gradient of METOH/H_2O/lithium perchlorate and ETOH/lithium perchlorate - [tocopherol, tocotrienol]	ODS, C18, 5 μm 250 × 4.6 mm PS: 5 μm, 5 RP_{18} pre-column 30 × 4.6 mm	none 0.2–0.3 pmol [> 90%]	Podda et al., 1996, 1999 [48, 62]
guinea pig (liver, adrenal gland, kidneys, brain—guinea pig, blood—human)	- homogenization in H_2O - extraction with hexane/ETOH - redissolution in ETOH	- Q_{ox} /Q_{red} : ECD and UVD λ = 275 nm - ϕM: sodium perchlorate/METOH/ETOH	RP, C18, 5 μm 150 × 4.0 mm	none Q_{ox} = 2 ng; Q_{red} = 0.1−0.15 ng [95%]	Ikenoya et al., 1981 [63]

(Continued)

SPECIES (Other tissues-species)	HOMOGENIZATION/ EXTRACTION	HPLC ANALYSIS AND[OTHER COMPONENTS ANALYZED SIMULTANEOUSLY]	COLUMN SPECIFICATIONS	INTERNAL STANDARD (External standard/standard addition) LIMIT OF DETECTION AND [RECOVERY]	REFERENCE
guinea pig (liver, kidney, myocardium)	- homogenization in Tris-HCl buffer - extraction with hexane - redissolution in propanol or ETOH	- Q_{ox}/Q_{red}: ECD and UVD λ = 275 nm - ϕM: sodium perchlorate/METOH/ETOH	RP, C18, 10 μm 250 × 4.6 mm	none Q_{ox} = 1 ng; Q_{red} = 0.1 ng n/a	Katayama et al., 1980 [64]
rabbit (muscle—human, rat, guinea pig, liver—rat, guinea pig, adipose tissue—rat, guinea pig, spinal cord—rabbit, blood—human, plasma—human, rat)	- homogenization in H_2O/BHT/SDS - (ascorbate added to blood) - extraction with hexane/ETOH - redissolution in METOH/ETOH	- Q_{ox}/Q_{red}: ECD and UVD λ = 275 nm - ϕM: lithium perchlorate/METOH/ETOH - [tocopherol]	ODS, 5 μm 250 ×4.6 mm	none 1 pmol [89%]	Lang et Packer, 1987; Lang et al., 1986 [31, 35]
dog, beef	- homogenization in acetone - extraction with dichloromethane - redissolution in ETOH	- UVD λ = 280 nm - ϕM: H_2O/ETOH	ODS 250 × 4.6 mm	Q_{11} 0.001 ng [94%]	Vadhanavikit (a) et al., 1984 [66]
B. MUSCLE					
human, guinea pig, rat (liver—human, guinea pig; adipose tissue—rat, guinea pig myocardium—rabbit; plasma and blood—human)	- homogenization in H_20/BHT - extraction with hexane/ETOH - redissolution in METOH/ETOH	- UVD λ = 275 nm - ϕM: METOH/ETOH - [tocopherol]	ODS, 5 μm 250 × 4.6 mm	(external standard) 50 nmol/L [89%]	Lang and Packer, 1987 [35]
rat, guinea pig (blood and plasma—rat and human; liver—rat and guinea pig)	- homogenization in H_2O/BHT/SDS - (ascorbate added to blood) - extraction with hexane/ETOH - redissolution in METOH/ETOH	- UVD λ = 275 nm - ϕM: METOH/ETOH - [tocopherol]	ODS, 5 μm 250 × 4.6 mm	(external standard) 0.1 μM [n/a]	Lang et al., 1986 [31]
C. LIVER					
rat	- homogenization in saline/BHT/SDS - extraction with hexane - redissolution in chloroform/METOH	- Q_{ox}/Q_{red}: ECD - ϕM: gradient of zinc chloride/ sodium acetate/METOH and zinc chloride/sodium acetate/ METOH/ETOH - [tocopherol and homologues]	RP, 18.5 μm 125 × 4 mm	none Q_{ox} = 1 pmol; Q_{red}: 0.6 pmol [95%]	Leray et al., 1998 [67]

rat (kidney, spleen, testes, brain)	- homogenization in METOH/H_2O - extraction with METOH/chloroform - redissolution in METOH/H_2O	- UVD λ = 214 nm - ϕM: gradient of METOH/H_2O and METOH/isopropanol - [dolicyl phosphate, dolichol, cholesterol]	ODS, 6 cm RP, 3 μm 60 × 4.6 mm	Q_6 0.1 ng (dolichol) [n/a]	Elmberger et al., 1989 [41]
guinea pig (mitochondria)	- homogenization in sucrose - pellet resuspended in Tris-HCl, pH 7.4 - extraction with hexane/ETOH - redissolution in ETOH or propanol	- Q_{ox}/Q_{red} : ECD and UVD λ = 275 nm - ϕM: sodium perchlorate/ METOH/ETOH	Nucleosil, C-18, 5 μm 150 × 4.0 mm	none n/a [92%]	Takada et al., 1982 [68]
dog	- homogenization and extraction with METOH/chloroform - redissolution in chloroform	- ECD λ = 272 nm - ϕM: isopropanol/heptane [triglycerides, cholesteryl ester, cholesterol, dolichol, tocopherol, retinol]	NP, CN 250 × 4.6 mm	none n/a [92%]	Greenspan et al., 1988 [69]
sheep	- extraction with chloroform/METOH - addition of BHT - redissolution in cyclohexane - decantation with acetone - redissolution in propanol/hexane	- UVD λ = 210 nm - ϕM: propanol/hexane - [dolichol, cholesterol]	CN, 10 μm 100 × 5 mm	none 50 ng [n/a]	Palmer et al., 1984 [70]
		D. BLOOD			
human	- mixing of blood with acetone - addition of EDTA - extraction with hexane/dichloromethane - redissolution with METOH/hexane	- UVD λ =280 nm - ϕM: METOH/hexane	ODS 250 × 4.6 mm	Q_{11} n/a [96%}	Morita and Folkers, 1993 [38]
human, rat, rabbit	- mixing of blood with acetone - extraction with dichloromethane - redissolution in ETOH	- UVD λ = 280 nm - ϕM: ETOH/H_2O	ODS 250 × 4.6 mm	Q_{11} 10 ng [94%]	Muratsu et al., 1988 [71]
human, rat (liver—rat)	- homogenization with saline - extraction with hexane/ETOH - redissolution in ETOH	- Q_{ox}/Q_{red}: ECD - ϕM: sodium perchlorate/ acetonitrile/METOH/ETOH	C18, 5 μm 250 × 4.6 mm	Q_{11} 0.15 ng [98%]	Okamoto et al., 1988 [72]

(Continued)

SPECIES (Other tissues-species)	HOMOGENIZATION/ EXTRACTION	HPLC ANALYSIS AND[OTHER COMPONENTS ANALYZED SIMULTANEOUSLY]	COLUMN SPECIFICATIONS	INTERNAL STANDARD (External standard/standard addition) LIMIT OF DETECTION AND [RECOVERY]	REFERENCE
human	- mixing of blood with ether/hexane (TLC) - extraction with dichloromethane - redissolution in ETOH	- UVD; λ = 280 nm - ϕM: H_2O/ETOH	ODS 250 × 4.6 mm	Q_{11} and 2,3,5 trimethyl-6-decaprenyl-1,4-benzoquinone 1μg [94%]	Vadhanavikit et al., (b), 1984 [73]
human (serum)	- extraction with hexane/ETOH - redissolution in isopropanol	- Q_{ox}/Q_{red}: ECD - ϕM: sodium perchlorate/ETOH	RP, C18, 10 μm 250 × 4.6 mm	TQ –10 0.150 ng [n/a]	Ikenoya et al., 1979 [74]
human	- extraction with hexane - redissolution in isopropanol	- UVD λ = 275 nm - ϕM: sodium perchlorate/ETOH	RP, C18, 10 μm 250 × 4.6 mm	TQ –10 0.2 μg [n/a]	Ikenoya et al., 1978 [75]
human (serum, liver—mouse)	- addition of pyrogallol as antioxidant - extraction with hexane - redissolution in dioxane	- UVD λ = 275 nm, or fluometric detection - ϕM: ETOH/H_2O	ODS/RP 500 × 4.6 mm	TQ-9 15 μg [98%]	Abe et al., 1978 [42]
E. PLASMA					
human	- mixing of plasma with ETOH - supernatant injected directly	- Q_{ox}/Q_{red} : ECD - ϕM: gradient of sodium perchlorate/METOH and ETOH/*t*-butyl alcohol	C18, 5 μm 125 × 4.0 mm	none 2.5 nmol/L [91%]	Wang et al., 1999 [76]
human (neonatal)	- extraction with hexane/ETOH - redissolution in METOH/ETOH/propanol	- Q_{ox}/Q_{red}: ECD - ϕM: lithium perchlorate/ METOH/ETOH/propanol - [carotenoids, tocopherol]	RP, C18, 5 μm 250 × 4.0 mm	none 7 nmol/L [97–105%]	Finchk et al., 1999 [46]
human	- addition of EDTA - extraction with hexane/ETOH - redissolution in hexane/ETOH	- UVD λ = 275 nm - ϕM: METOH/hexane	C-8, 5 μm 250 × 4.6 mm	none 25 ng/mL [78%]	Graves et al., 1999 [61]
human	- extraction with hexane/METOH - redissolution in hexane	- Q_{ox}/Q_{red}: UVD λ = 284 nm - ϕM: METOH/hexane	RP, C-18, 5 μm 250 × 4.0 mm	none 0.05 – 0.15 ppm [n/a]	Karpinska et al., 1998 [77]

human	- extraction with petroleum ether/METOH/H_2O - redissolution in METOH/chloroform - addition of $NaBH_4$	- Q_{ox}/Q_{red}: ECD and UVD λ = 275 nm - ϕM: gradient of lithium perchlorate/METOH/H_2O and lithium perchlorate/ETOH/propanol/hexane - [tocopherol]	RP, C-18, 3 μm 100 × 4.6 mm	(standard addition) n/a [95%]	Zhang et al., 1998 [47]
human	extraction with hexane/METOH - organic phase injected directly	- Q_{ox}/Q_{red}: ECD and UVD λ = 210 nm - ϕM: METOH/*t*-butyl alcohol	LC-8, 5 μm 250 × 4.6 mm	none 4 nmol/L [99%]	Yamashita and Yamamoto 1997 [39]
human (with coronary artery diseases)	- oxidation of serum with $CuSO_4$ - addition of EDTA - extraction with propanol	- Q_{ox}/Q_{red}: ECD and UVD λ = 275 nm - ϕM: sodium perchlorate/METOH/propanol	n/a	none n/a [n/a]	Lagendjik et al., 1997 [15]
human	- addition of EDTA - extraction with propanol - organic phase injected directly	- Q_{ox}/Q_{red}: ECD and UVD λ = 275 nm - ϕM: sodium perchlorate/METOH/propanol	RP, ODS2 50 mm × 4.6 mm	none n/a [n/a]	Lagendijk et al., 1996 [34]
human	- addition of heparin - extraction with hexane/METOH - dissolution with propanol/METOH	- UVD λ = 275 nm - ϕM: METOH/hexane	RP, C-18, 5 μm 150 × 3 mm	(standard addition) 90 ng/mL [64%]	Kaplan et al., 1995 [37]
human [neonatal]	- extraction with hexane/ETOH - redissolution in METOH/ETOH	- Q_{ox}/Q_{red}: ECD - ϕM: lithium perchlorate/METOH/ETOH/propanol - [carotenoids, tocopherol]	RP, C-18, 5 μm 250 × 4 mm	none 7 nmol/L [97%]	Finckh et al., 1995 [53]
human [healthy subjects, athletes, hyper- and hypothyroid, and hypercholesterolemic patients]	- extraction with hexane/METOH - redissolution in propanol	- $Q_{ox}Q_{red}$: ECD and UVD λ = 275 nm - ϕM: sodium acetate/propanol/METOH	RP XL C-18, 3 μm 70 × 4.6 mm	(standard addition) UV: 50 ng/mL, ECD: 5 ng/mL [87%]	Grossi et al., 1992 [50]
human	- extraction with hexane/METOH/SDS - (effect of SDS concentration)	- UVD λ = 275 nm - ϕM: ETOH/H_2O	RP, ODS, 5 μm 150 × 4.6 mm	none n/a [95%]	Hirota et Kawase, 1984 [44]
dog	- mixing of plasma with trichloroacetic acid - extraction with hexane - elution with hexane/METOH: hexane (silica cartridge)	- UVD λ = 275 nm - ϕM: METOH/hexane	RP, C-18, 4 μm 150 × 3.9 mm	(standard addition) 50 ng/mL [93%]	Kommuru et al., 1998 [65]

(Continued)

SPECIES (Other tissues-species)	HOMOGENIZATION/ EXTRACTION	HPLC ANALYSIS AND[OTHER COMPONENTS ANALYZED SIMULTANEOUSLY]	COLUMN SPECIFICATIONS	INTERNAL STANDARD (External standard/standard addition) LIMIT OF DETECTION AND [RECOVERY]	REFERENCE
		F. SUBCELLULAR FRACTIONS			
human, rat (liver)	- homogenization in sucrose (photoprotection) - extraction with METOH/ether - redissolution in hexane - reduction (post-extraction) with $NaBH_4$	- $Q_{ox/}Q_{red}$: UVD λ = 210 nm - ϕM: gradient of METOH/H_2O and METOH/propanol/hexane	RP, ODS, 3 μm	none n/a [n/a]	Åberg et al., 1996 [78]
rat (liver, blood, kidney, lung, brain, skeletal muscle)	- homogenization in sucrose - pellet resuspended in Tris-HCl - extraction with hexane/ETOH	- $Q_{ox/}Q_{red}$: ECD - ϕM: sodium perchlorate/ acetonitrile/METOH/ETOH	n/a	none n/a [n/a]	Takahashi et al., 1993 [79]
rat, beef (liver, heart)	- homogenization in sucrose - extraction with METOH/chloroform at 37°C (2 × 1 hr) - redissolution in chloroform	- UCD λ = 275 nm and 210 nm - ϕM: hexane/ether/acetic acid - gradient of METOH/H_2O and METOH/isopropanol	RP, ODS, 3 μm	none n/a [n/a]	Kalén et al., 1987 [21]
rat (liver, mitochondria)	- homogenization in H_2O/BHT/SDS/or in sucrose - (ascorbate added to blood) - extraction with hexane/ETOH - redissolution in METOH/ETOH	- Q_{ox}/Q_{red}: ECD and UVD λ = 275 nm - ϕM: lithium perchlorate/ METOH/ETOH - [tocopherol]	ODS, 5 μm 250 × 4.6 mm	(external standard) 2 pmol [84%]	Lang et al., 1986 [31]

Note: ϕM = mobile phase; λ = wavelength; BHT = butylated hydroxytoluene; CN = cyanopropyl; ECD = electrochemical detection; EDTA = ethylenediaminetetraacetic acid; ETOH = ethyl alcohol; HPLC = high-performance liquid chromatography; METOH = methyl alcohol; ppm = parts per million (10^{-6}); PS = particle size; RP = reversed phase; NP = normal phase Q = ubiquinone (i.e. = Q_{red} + Q_{ox}); Q_{red} = ubiquinol (reduced form of ubiquinone); Q_{ox} = ubiquinone (oxidized form); SDS = sodium dodecylsulfate; UVD = ultra-violet detection.

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Part 3

Health Effects of Coenzyme Q

Section 3A

Coenzyme Q Status and Oxidative Stress In Vivo

16 Plasma Ubiquinol-10 as a Marker for Disease

Anatol Kontush

CONTENTS

16.1 INTRODUCTION

Ubiquinone-10, also known as coenzyme Q10, is best known for its role in energy production by mitochondria, where it functions as an essential proton-electron carrier in the inner mitochondrial membrane.[1] The human body contains about 1.6 g ubiquinone-10 which is present in nearly all tissues.[2] Ubiquinol-10, the reduced form of ubiquinone-10, is a potent lipophilic antioxidant for protection of lipids in different biological and model systems.[3–5] Ubiquinol-10 represents more than 80% of the total coenzyme Q10 (ubiquinol-10 + ubiquinone-10) pool in human plasma and liver[6] and is accordingly an important antioxidant in plasma lipoproteins [7–9] and hepatocytes.[10,11]

The high antioxidative efficiency of ubiquinol-10 is closely related to its extreme sensitivity to oxidation. Ubiquinol-10 is easily oxidized to ubiquinone-10 both *in vivo* and *in vitro*.[7–9,12] Exceptional oxidative lability of ubiquinol-10 implies that *in vivo* it is expected to be oxidized before other antioxidants, as is the case *in vitro* during lipoprotein[7–9] and hepatocyte[10,11] oxidation. Lipid peroxidation is strongly implicated as playing an important role in the development of various pathologies, such as some cardiovascular and neurological diseases.[13] Oxidation of plasma lipoproteins (where most of the blood ubiquinol-10 is located)[2] appears to represent a crucial step in atherogenesis and is also likely to occur in other diseases linked to increased free radical production.[14] All of these pathologies might, therefore, be associated with a decreased plasma level of ubiquinol-10.

Most of ubiquinol-10 in human plasma appears to be produced in the liver.[15,16] Hepatocytes can efficiently synthesize ubiquinol-10 via the mevalonate pathway.[17] They can produce it through the reduction of both endogenous and exogenous ubiquinone-10.[8,16] This implies that liver deficiency might also result in a decreased plasma level of ubiquinol-10. Taken together, these findings suggest that measurement of ubiquinol-10 in human plasma might serve as a marker for some diseases associated with increased oxidative stress and/or liver deficiency.

Due to its high instability, reliable measurement of ubiquinol-10 is not a trivial task. For many years after its discovery in 1957,[18] quantification of its total (reduced + oxidized) level was the

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only practical approach to measuring coenzyme Q10 in clinical studies. Simple and clinically applicable methods for the measurement of ubiquinol-10 have only been developed in the last few years.[19–23] Since then, a number of studies have been published that characterize the level of ubiquinol-10 in different diseases. Most often, the plasma or whole blood concentration of ubiquinol-10 has been measured.

This review is aimed at assessing the hypothesis that the plasma level of ubiquinol-10 may represent a new disease marker. The results of the studies on plasma ubiquinol-10 will be summarized and compared with the earlier data on the plasma level of total coenzyme Q10.

16.2 CARDIOVASCULAR DISEASES

Since oxidation of plasma lipoproteins, primarily low density lipoprotein (LDL), is thought to represent a key step in the development of atherosclerosis,[14] decrease in plasma and/or LDL levels of ubiquinol-10 can be expected to occur in atherosclerotic patients. Several studies have been recently performed to test this hypothesis (Table 16.1).

No difference in absolute plasma concentration of ubiquinol-10 (expressed as mass/volume) between patients with coronary artery disease (CAD) and healthy controls has been observed in any of the studies,[24–27] including those performed in our laboratory,[26,27] nor has any decrease in plasma ubiquinol-10 in CAD been found when its level is normalized to lipids or expressed as a percentage of total coenzyme Q10.[24,26,27] Similarly, no difference in levels of ubiquinol-10 in LDL of patients vs. controls has been observed.[24] The only study where a significant decrease in plasma ubiquinol-10 in CAD has been reported is that of Lagendijk et al.[25] However, this difference is seen only when ubiquinol-10 is expressed as its ratio to ubiquinone-10, is moderate, and comprises about 0.5% when recalculated to a percentage of total coenzyme Q10. A comparable, but insignificant trend toward lower values of ubiquinol-10 in CAD has been observed in other studies[24,26,27] where fewer subjects (than in [25]), were investigated. This is also in accordance with lower concentrations of total coenzyme Q10 reported for CAD patients.[28] The trend to lower plasma ubiquinol-10 is accompanied by a considerable overlapping of values between the patient and control groups.[24,26,27]

These data can be summarized in such a way that any reduction in plasma level of ubiquinol-10 in atherosclerosis is minor, indicating that this parameter only weakly depends on lipoprotein oxidation in the arterial wall and does not represent a marker for atherosclerosis in humans.

TABLE 16.1
Plasma Ubiquinol-10 in Cardiovascular Diseases

Disease	Level In	Difference Compared to Controls	Ref.
Coronary artery disease	Plasma	No difference[a, b, c]	[24]
	LDL	No difference[a, b, c]	
	Plasma	No difference[a]	[25]
		Lower[d]	
	Plasma	No difference[a, b, c]	[26]
		No difference[a, b, c]	[27]

[a] Absolute concentration.
[b] lipid-normalized concentration.
[c] percentage of ubiquinol-10 + ubiquinone-10.
[d] ratio to ubiquinone-10.

16.3 HYPERLIPIDEMIA, DIABETES, AND OTHER METABOLIC DISEASES

Hyperlipidemia is a metabolic dysfunction that increases the risk of atherosclerosis and can also, therefore, be related to the increased lipoprotein oxidation and lower plasma levels of antioxidants. However, absolute concentrations of ubiquinol-10 in plasma of hyperlipidemic patients tend to be elevated (Table 16.2).[26,29,30] This is in accordance with higher values of plasma lipids in hyperlipidemia, since ubiquinol-10, as a highly lipophilic substance, is transported in lipoproteins and therefore its level correlates with that of lipids.[2,31] It should be taken into account, that in order to provide sufficient protection of lipids against oxidation, the amount of a lipophilic antioxidant must be the same in normo- and hyperlipidemic subjects when calculated per mass unit of lipids. This emphasizes that lipid-normalized, rather than absolute values of plasma ubiquinol-10 must be examined when two groups of subjects are compared.

When this comparison is performed for hyper- and normolipidemic subjects, lipid-normalized ubiquinol-10 is significantly lower in the patients than in controls.[26] Since ubiquinol-10 is transported in all plasma lipoproteins[2,6] and is essential for the protection of all of them against oxidation,[5] it is important to normalize it either to total lipids or to the sum of total cholesterol and triglycerides, the major plasma lipids. This probably explains why plasma levels of ubiquinol-10 have been found to be similar in hyperlipidemic patients and healthy subjects when normalized only to total cholesterol.[29,30] This assumption is in line with the fact that when plasma ubiquinol-10 is expressed as a percentage of total coenzyme Q10[26] or as a ratio to ubiquinone-10[30] (i.e., independently of plasma lipids), it is significantly lower in hyperlipidemic subjects than in control. The ratio of ubiquinol-10 to ubiquinone-10 is also significantly lower in LDL isolated from hyperlipidemic patients in comparison to control subjects.[30] Similarly, the ubiquinol-10/ubiquinone-10 ratio is significantly lower in plasma of hyperlipidemic, than normolipidemic, rats.[32]

To assess whether factors, other than hyperlipidermia, were important determinants of plasma ubiquinol-10, we performed multiple regression analysis using age, sex, body mass index, smoking, and presence of different diseases as independent factors.[26] We found that smoking was the most important determinant of plasma levels of ubiquinol-10 in our study population. Low ubiquinol-10

TABLE 16.2
Plasma Ubiquinol-10 in Hyperlipidemia, Diabetes, and Other Metabolic Diseases

Disease	Level In	Difference Compared to Controls	Ref.
Hyperlipidemia	Plasma	No difference[b]	[29]
	Plasma	No difference[a, b]	[30]
		Lower[d]	
	LDL	No difference[b]	
		Lower[d]	
	Plasma	Higher[a]	[26]
		Lower[b, c]	
Diabetes	Plasma	Lower[b]	[29]
	Plasma	Lowerrt[a, b, c]	[41]
Homocystinuria	Plasma	Higher[d]	[42]

[a] Absolute concentration.
[b] lipid-normalized concentration.
[c] percentage of ubiquinol-10 + ubiquinone-10.
[d] ratio to ubiquinone-10.

values were also observed in subgroups of subjects with hypertension, liver disease, and increased alcohol consumption.

Treatment with statins (inhibitors of 3-hydroxy-3-methylglutaryl coenzyme *A* reductase), which are routinely used to decrease plasma cholesterol in hyperlipidemic patients, is another factor that can result in decreased plasma levels of ubiquinol-10. Both cholesterol and ubiquinol-10 are synthesized in the liver via the common mevalonate pathway, and inhibition of its key enzyme can lead to a decrease of both in plasma. A decrease in total plasma coenzyme Q10 has been reported following statin treatment.[33–39] Remarkably, the coenzyme Q10/cholesterol ratio is often decreased as a result of the treatment,[34,38] indicating that the synthesis of ubiquinol-10 might be more severely affected by statins than that of cholesterol.

Diabetes, another metabolic dysfunction related to increased oxidative stress,[40] has also been reported to result in low plasma levels of ubiquinol-10 (Table 16.2).[29] Although we have recently confirmed this finding,[41] when multiple regression analysis on plasma ubiquinol-10 values was performed using the presence of different diseases as independent factors, significantly lower levels of this antioxidant were only associated with the presence of liver dysfunction. No such analysis was performed in [29], suggesting that factors other than diabetes may have been responsible for the decreased levels of ubiquinol-10 found.

Plasma ubiquinol-10 has also been measured in homocystinuria, a metabolic disease characterized by increased accumulation of homocysteine which is a risk factor for atherosclerosis (Table 16.2).[42] No expected decrease in ubiquinol-10 has been found in the patient plasma compared to healthy controls. In fact, ubiquinol-10 was even increased in homocystinuria, once again demonstrating the absence of the association between its low plasma values and atherosclerosis (Table 16.1).

Together, these data suggest that although plasma ubiquinol-10 is decreased in metabolic dysfunctions such as hyperlipidemia and diabetes, it is unclear whether this decrease is primary, due to these pathologies, or secondary, due to other confounding factors.

16.4 NEUROLOGICAL DISEASES

Pathological oxidation is a common mechanism, playing a role in the development of such neurological diseases as Alzheimer's disease,[43] Parkinson's disease,[44] and others.[45] Since neurological targets of oxidation are thought to be located in the brain, only a few studies have been performed measuring oxidation parameters in plasma of affected subjects. We have recently shown that oxidation of lipoproteins present in cerebrospinal fluid may be an important event in the pathogenesis of Alzheimer's disease.[46] This suggests that plasma lipoproteins might also be oxidatively modified in this disease. However, we found no significant decrease in ubiquinol-10 in plasma of patients with Alzheimer's disease in comparison with corresponding age-matched controls, independently of how it was expressed (Table 16.3). Nor was any difference in plasma ubiquinol-10 found between controls and patients with amyotrophic lateral sclerosis[47] or Parkinson's disease.[48]

The only neurological disease that has been reported to be related to significantly lower values of ubiquinol-10 compared to controls, is mevalonic aciduria, which is caused by a defect in the mevalonate kinase gene.[49] This disease results in a decrease in LDL ubiquinol-10 during crisis conditions. However, the effect is unlikely to be of a neurological origin and can be ascribed to the fact that mevalonate kinase is directly involved in the biosynthesis of ubiquinol-10. These data indicate that plasma ubiquinol-10 is not decreased in chronic neurological diseases and cannot therefore serve as a marker for them.

16.5 LIVER DISEASES

Since most ubiquinol-10 in human plasma originates in the liver,[16] liver diseases can lead to a decrease in its plasma level. Indeed, ubiquinol-10 has been found to be significantly lower in plasma of patients with liver diseases such as hepatitis, cirrhosis, and hepatic carcinoma when compared

TABLE 16.3
Plasma Ubiquinol-10 in Neurological Diseases

Disease	Level In	Difference Compared to Controls	Ref.
Alzheimer's disease	Plasma	No difference[a, b, c]	[51]
		No difference[a, b, c]	[46]
Parkinson's disease	Plasma	No difference[a, b, c]	[48]
Mevalonic aciduria	LDL	Lower[b]	[49]
Amyotrophic lateral sclerosis	Plasma	No difference[a]	[47]

[a] Absolute concentration.
[b] lipid-normalized concentration.
[c] percentage of ubiquinol-10 + ubiquinone-10.

TABLE 16.4
Plasma Ubiquinol-10 in Liver Diseases

Disease	Level In	Difference Compared to Controls	Ref.
Chronic active hepatitis	Plasma	Lower[c]	[50]
Cirrhosis	Plasma	Lower[c]	[50]
Hepatocellular carcinoma	Plasma	Lower[c]	[50]
Liver dysfunction[d]	Plasma	No difference[a, b]	[51]
		Lower[c]	

[a] Absolute concentration.
[b] lipid-normalized concentration.
[c] percentage of ubiquinol-10 + ubiquinone-10.
[d] hepatits or cirrhosis.

to controls (Table 16.4).[50,51] These results are in accordance with low values of total coenzyme Q10 in plasma of patients with cirrhosis reported by others.[52] Plasma ubiquinol and total coenzyme Q are also decreased as a result of hepatic injury in animal models.[53–55] All these data support an important role for the liver in the redox metabolism of ubiquinol-10.

Interestingly, ubiquinol-10 was lower in patients with liver disease only when expressed as a percentage of total ubiquinol-10 + ubiquinone-10. In contrast, lipid-normalized ubiquinol-10 tended to be higher in patients with hepatitis and cirrhosis.[51] This suggests that if liver dysfunction results in its impaired capacity to reduce ubiquinol-10, synthesis of this antioxidant might be upregulated in order to overcome the lower extent of reduction. Together, these results demonstrate that plasma ubiquinol-10, expressed as a percentage of total coenzyme Q10, is decreased in the presence of liver dysfunction and may therefore be used as a marker for it.

16.6 OTHER DISEASES

Plasma levels of ubiquinol-10 have been measured in several other diseases and pathological conditions. Significantly lower values of plasma ubiquinol-10 (compared to controls), have been reported for adult respiratory distress syndrome[56] and infant asphyxia.[57] Both these conditions are related to highly increased oxidative stress. This implies that plasma ubiquinol-10 can be decreased as a result of a massive and acute oxidative stress when antioxidant defense systems of the body are overcome. It seems that in order to cause a detectable decrease in plasma ubiquinol-10, oxidative stress must be extensive, as is the case for the pathologies mentioned above. This is in line with

the observation that plasma ubiquinol-10 is not reduced in smoking, i.e., under conditions of milder and chronic oxidative stress.[22]

16.7 CONCLUSIONS

Taken together, currently available data indicate that decreased levels of ubiquinol-10 in human plasma may have two major causes: the presence of liver dysfunction (hepatitis, cirrhosis, hepatoma) and/or highly increased systemic oxidative stress (adult respiratory distress syndrome, infant asphyxia). Low plasma ubiquinol-10 levels might also reflect less pronounced oxidative stress in hyperlipidemia and diabetes. However, they may also (at least partly) be related to confounding liver dysfunction known to occur in hyperlipidemia[58] and frequently present in diabetes. The latter explanation seems to be more probable, since comparable oxidative stress chronically present in atherosclerosis or neurological diseases such as Alzheimer's or Parkinson's disease, is unable to cause a decrease in plasma ubiquinol-10.

Ubiquinol-10 synthesis by the liver and its consumption by oxidative processes accordingly represent the major determinants of its plasma level (Figure 16.1). Typically, liver synthesis seems to be more important, and efficient recycling of ubiquinol-10 by this organ appears to outweigh its increased consumption by oxidants under conditions of relatively mild and chronic oxidative stress. It seems that the human body can efficiently maintain ubiquinol-10 levels in the absence of liver dysfunction. Conditions of acute oxidative stress are likely to be the only exception, when the efficiency of the ubiquinol-synthesizing systems of the liver is not sufficient to compensate for its consumption and to maintain its plasma level.

This suggests that measurement of plasma ubiquinol-10 can be diagnostically used to assess the function of the liver. It remains to be shown whether measuring ubiquinol-10 can provide additional information in comparison with established liver markers. In the absence of liver dysfunction, low plasma ubiquinol-10 might be a marker for a massive and acute oxidative stress. In contrast, plasma level of ubiquinol-10 is not indicative of the presence of cardiovascular and neurological diseases related to milder and chronic oxidative conditions, such as atherosclerosis, Alzheimer's, or Parkinson's disease.

Finally, it must be emphasized that the way that the plasma level of ubiquinol-10 is expressed is critically important for its value as a disease marker. Plasma level of ubiquinol-10 can be expressed

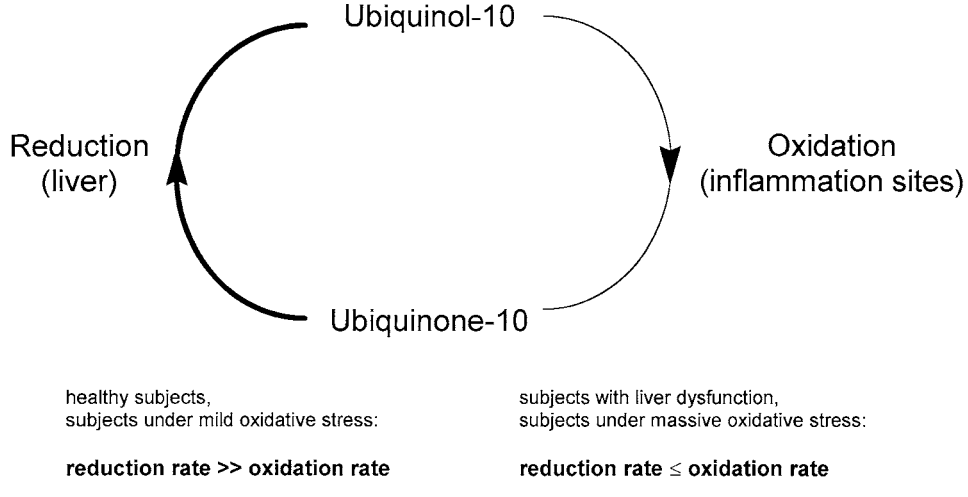

FIGURE 16.1 Major pathways of the metabolism of ubiquinol-10 in human plasma and their relationship in health and disease. The physiologically predominant reduction pathway is shown as a bold line.

as its absolute concentration (mass of ubiquinol-10/volume of plasma), lipid-normalized concentration (mass of ubiquinol-10/mass of plasma lipids), percentage of total coenzyme Q10 (mass of ubiquinol-10/mass of ubiquinol-10 plus ubiquinone-10), or as a ratio to the level of its oxidized form (mass of ubiquinol-10/mass of ubiquinone-10). Since ubiquinol-10, as a lipophilic antioxidant, is transported in lipoproteins, its absolute plasma level often reflects that of plasma lipids. Differences in the metabolism of ubiquinol-10 between two groups of subjects may therefore be masked by differences in plasma lipids, when its absolute plasma levels are compared. This means that plasma concentrations of ubiquinol-10 must be normalized to the lipids rather than used without such a normalization. This is essential when groups with very different levels of plasma lipids, such as hyper- and normolipidemic subjects, are compared. Our experience shows that lipid normalization can reveal differences in lipophilic antioxidants that are hardly detectable using their absolute concentrations.[27] Calculation of the plasma level of ubiquinol-10 as a percentage of total coenzyme Q10 (or as a ratio to the level of its oxidized form) represents an even more sensitive approach to detect abnormalities in its metabolism, which often remain undetectable using absolute or lipid-normalized concentrations (e.g., for liver dysfuction).[50,51] Taken together, these data point out that in order to serve as a diagnostic marker, plasma level of ubiquinol-10 should be expressed in two different ways: normalization to the level of plasma lipids and to the level of total coenzyme Q10.

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17 Redox Status of Plasma Coenzyme Q as an Indicator of Oxidative Stress

Yorihiro Yamamoto and Satoshi Yamashita

CONTENTS

17.1 INTRODUCTION

Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favor of the former[1] and has been suggested to be a causative factor in aging and in degenerative diseases such as heart attack, diabetes, and cancer. One can measure oxidative stress by detecting oxidation products of lipids, proteins, and DNA. However, the ratio of the oxidized form of redox compound to its reduced form should give us more direct figures.

To find oxidatively vulnerable antioxidants (reduced form of redox compounds), we incubated human plasma in the presence or absence of 5 μM cupric ion under aerobic conditions at 37°C.[2] Figure 17.1 shows that ascorbate was depleted first and followed by the depletion of ubiquinol-10 (reduced form of coenzyme Q, $CoQH_2$-10). However, no significant decrease in α-tocopherol level was observed. Despite the presence of α-tocopherol (VE), formation of cholesteryl ester hydroperoxide (CE−OOH) was observed after 46 h incubation. The addition of 5 μM cupric ion accelerated the depletion of ascorbate and ubiquinol-10 and the formation of CE−OOH. However, α-tocopherol remained almost unchanged. These results do not mean α-tocopherol is not a good antioxidant. As we observed that the oxidation of a lipid microsphere consisting of phosphatidylcholine and cholesteryl ester is inhibited efficiently by ascorbate or ubiquinol-10 in the presence of α-tocopherol but not in the absence of α-tocopherol (unpublished data), the presence of

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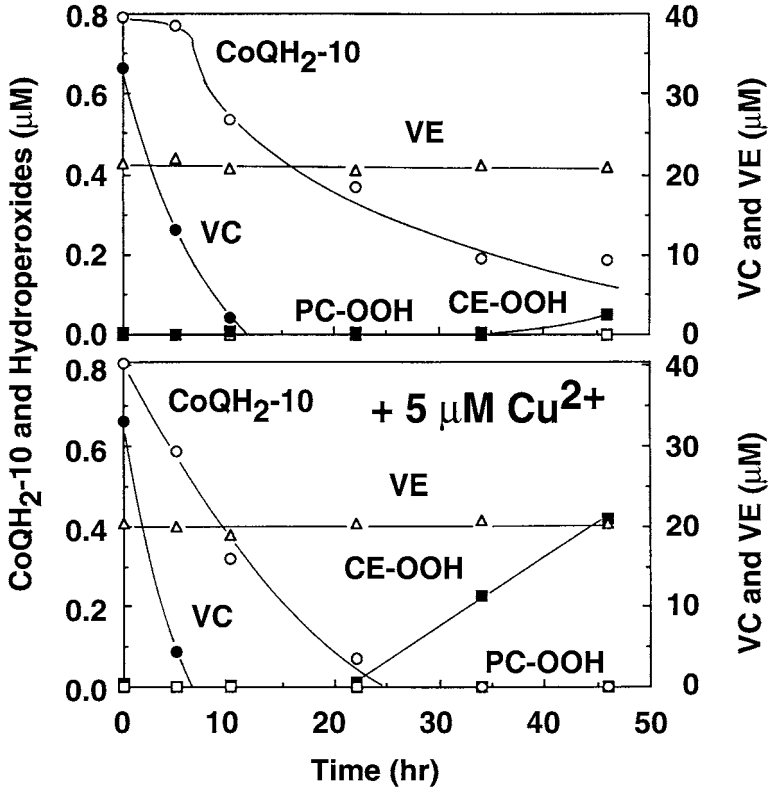

FIGURE 17.1 Changes in levels of ascorbate (VC), ubiquinol-10 ($CoQH_2$-10), α-tocopherol (VE), phosphatidylcholine hydroperoxide (PC–OOH), and cholesteryl ester hydroperoxide (CE–OOH) during the oxidation of heparinized human plasma in the absence (upper panel) or presence (lower panel) of 5 μM cupric chloride ($CuCl_2$) under aerobic conditions at 37°C. Initial concentrations of free cholesterol, cholesterol esters, and phosphatidylcholine were 1.10, 2.47, and 2.48 mM, respectively. From [2] with permission.

α-tocopherol is essential. It has been shown that the α-tocopherol radical can propagate lipoprotein oxidation and that this can be prevented by the addition of ascorbate and ubiquinol-10.[3]

Both ascorbate and ubiquinol-10 are very sensitive to oxidation as shown in Figure 17.1. We selected plasma ubiquinol-10 and ubiquinone-10 (oxidized form of coenzyme Q, CoQ-10) as a marker of oxidative stress since ascorbate levels should be dependent on the amount of intake and as ascorbate is expected to be excreted readily.

17.2 METHODS

17.2.1 HPLC Systems and Conditions

Here we describe a simple and reproducible HPLC method[4] for simultaneous detection of plasma ubiquinol-10 and ubiquinone-10 using an on-line reduction column and an electrochemical detector (ECD). Reduction column is essential since ubiquinone-10 is insensitive to ECD.

Figure 17.2 shows the HPLC system consisting of an injector (model 7125, Rheodyne, Cotati, CA), a pump (Model LC-10AD, Shimadzu), two guard columns (Type Supelguard LC-ABZ, 5 μm, 20 $\times$ 4.6 mm i.d., Supelco Japan, Tokyo), an analytical column (Type Supelcosil LC-8, 5 μm, 250 $\times$ 4.6 mm i.d., Supelco Japan), a reduction column (Type RC-10-1, Irica, Kyoto), a UV detector (Model SPD-10A, Shimadzu), and an amperometric ECD (Model Σ 985, Irica). The UV detector was monitored

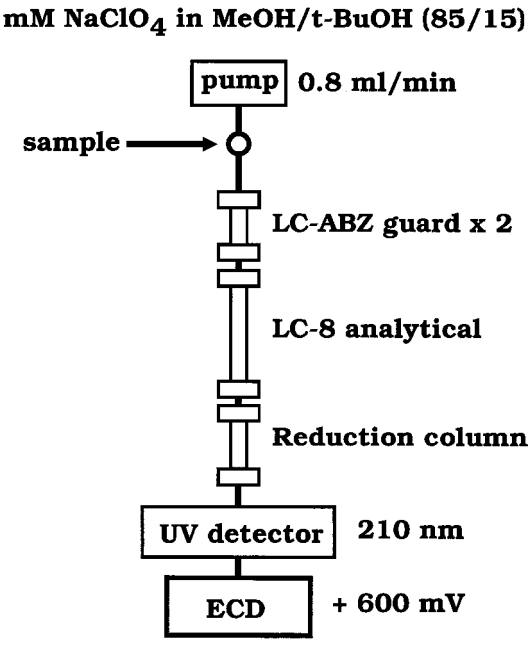

FIGURE 17.2 HPLC system for the detection of plasma ubiquinol-10 and ubiquinone-10.

at 210 nm and the oxidation potential for ECD was 600 mV. The mobile phase was 50 mM sodium perchlorate in methanol/tert-butyl alcohol (85/15, v/v) with a flow rate of 0.8 ml/min.

17.2.2 Analytical Procedure

Human heparinized plasma (50 μl) was mixed vigorously with 250 μl of methanol and 500 μl of hexane in a 1.5 ml-polypropylene tube. After centrifugation at 10,000 $\times$ g for 3 min at 4°C, 5 μl of hexane layer (corresponding to 0.5 μl of plasma) was injected immediately and directly for HPLC analysis.

The addition of tert-butyl alcohol to the mobile phase shortens the retention times of the above compounds and helps the solubility of hexane in the mobile phase because hexane is not very soluble in methanol. When the injection volume of hexane solution was greater than 10 μl, each peak became broad and/or two peaks (data not shown) due to higher solubilities of the above compounds in hexane than those in the mobile phase. Therefore, we fixed the injection volume of hexane solution as 5 μl.

A good linear relationship between the peak area and the injected amounts of ubiquinol-10 and ubiquinone-10 in the range of 0.05 to 20 pmol was observed (data not shown). The detection limit of ubiquinol-10 and ubiquinone-10 is about 2 fmol as judged by S/N ratio. Since 5 μl of hexane extract is equivalent to 0.5 ml plasma (see Methods), the detection limit of plasma ubiquinol-10 and ubiquinone-10 is about 4 (= 2/0.5) nM.

17.2.3 Application to Human Plasma

Figure 17.3A shows a chromatogram of the same sample monitored at 210 nm, indicating that free cholesterol (FC), cholesteryl arachidonate (Ch20:4), cholesteryl linoleate (Ch18:2), and cholesteryl oleate (Ch18:1) can be also quantified. The ABZ guard columns are necessary to separate tocopherols and free cholesterol.

Figure 17.3B shows a typical ECD chromatogram of the hexane extract from plasma of a healthy donor. Peaks were identified as tocopherols (mostly α-tocopherol), lycopene, β-carotene, ubiquinol-10, and ubiquinone-10 since the voltametric response ratios of each peak were the same as authentic samples.

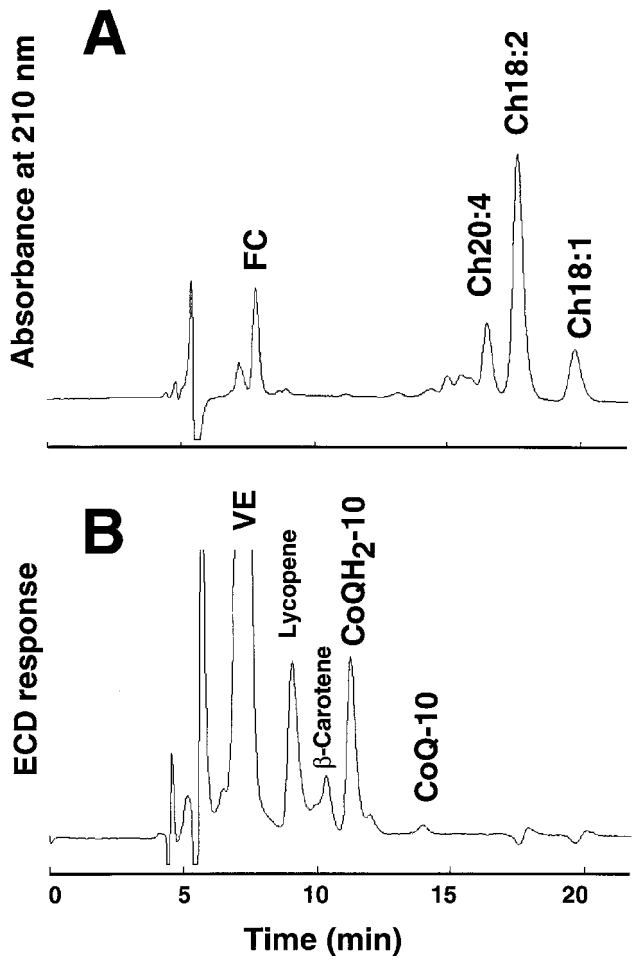

FIGURE 17.3 Typical HPLC chromatograms of hexane extract from a normal subject monitored by a UV detector (A) and an amperometric ECD (B). From [4] with permission.

17.2.4 Stability of Ubiquinol in the Extract

Stability of plasma VE and ubiquinol-10 in the hexane extract was measured at room temperature, 0 (on ice), −20, and −78°C. Ubiquinol-10 in the extract was stable only at −78°C. The rate of oxidation of ubiquinol-10 to ubiquinone-10 increased with increasing storage temperature. On the other hand, VE was stable at all conditions. These results clearly indicate that the hexane extract should be injected onto HPLC immediately after the extraction.

17.2.5 Recovery and Reproducibility

To verify the extraction efficiency, 1 volume of plasma (or distilled water) was mixed with 5 volumes of methanol and 10 volumes of hexane containing 0.58 μM α-tocopherol, 0.06 μM lycopene, 0.11 μM β-carotene, 0.20 μM ubiquinol-10, and 0.14 μM ubiquinone-10. HPLC analysis of the hexane phase revealed that all these lipid-soluble compounds were recovered at the hexane phase in a high yield, suggesting that they are stable under the procedure conditions described and exclusively partitioned into the hexane phase. The method is very reproducible but the coefficient of variation value for ubiquinone-10 was relatively high due to its low concentration in human plasma.

TABLE 17.1
Plasma Levels of Cholesterol and Antioxidants in Healthy Humans (male, $n = 31$, age = 29.1 ± 7.3)

Substance	Mean ± SD
FC (mM)	1.14 ± 0.23
CE (mM)	3.28 ± 0.64
Vitamin C (μM)	41.6 ± 32.7
Uric acid (μM)	370 ± 52
Vitamin E (μM)	21.8 ± 7.5
Lycopene (μM)	0.45 ± 0.30
β-carotene (μM)	0.99 ± 0.86
$CoQH_2$-10 (nM)	704 ± 205
CoQ-10 (nM)	32 ± 13
$CoQH_2$-10 +CoQ-10 (nM)	737 ± 275
%CoQ-10	4.5 ± 1.3

%CoQ-10 (%) = CoQ-10/($CoQH_2$-10 + CoQ-10)

17.2.6 Frozen Plasma

Plasma levels of lipid-soluble compounds including ubiquinol-10 and ubiquinone-10 in fresh plasma and twice frozen and thawed plasma were compared. Almost identical measurements were obtained between these two groups, indicating that frozen plasma samples can be used for the analysis of ubiquinol-10 and ubiquinone-10.

17.2.7 Plasma %CoQ-10 Value in Young Normal

Table 17.1 summarizes the results obtained from 31 male donors in the range of 22 to 45 years of age. It was found that percentages of the oxidized form of coenzyme Q-10 (%CoQ-10) in total coenzyme Q-10 is 4.5%,[5] indicating that plasma coenzyme Q-10 exists mostly in the reduced form.

17.3 APPLICATION

17.3.1 Oxidative Stress in Newborn Babies

Infants have less protection against oxidation since they have lower levels of antioxidants and antioxidant enzymes than healthy adults.[5] In addition, a rapid perfusion of oxygen in infants at birth may cause an increase of oxidative stress since ischemia/reoxygenation is considered as one of the major causes of oxidative stress.[6] In fact, infants have higher plasma level of F_2-isoprostanes,[7] free radical oxidation products of arachidonic acid, than adults. To obtain further evidence of oxidative stress in newborn infants we measured daily change in the redox status of plasma coenzyme Q using above described methods.

Blood was collected with an aliquot of heparin from the umbilical cord vein of infants at time of delivery and at 1, 3, and 5 days of age with parental consent. In this study samples were collected from 20 infants born normally at full term (gestational age was between 37 to 41 weeks) having body weights of 2345 to 3940 g. Nine infants were born with asphyxia (gestational age was from 31 to 41 weeks) having body weights of 1508 to 3352 g with lower than 5 points in their Apgar scores.

In normally born infants, plasma levels of vitamin C (the most reactive antioxidant in plasma as shown in Figure 17.1) decreased significantly after birth as shown in Figure 17.4A. Infants with asphyxia showed a similar decline in vitamin C levels yet a significant difference remained between the two groups at days 3 and 5.

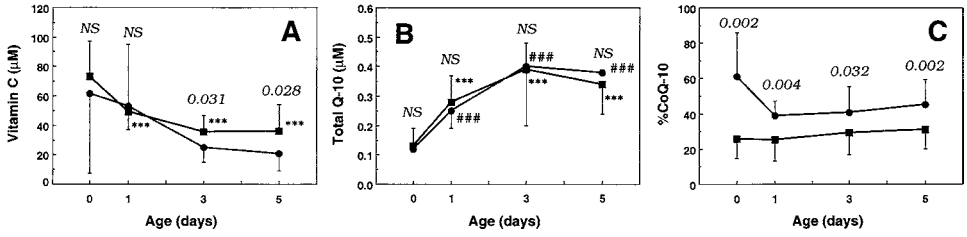

FIGURE 17.4 Changes in plasma levels of vitamin C (A), total coenzyme Q-10 (total Q-10) (B), and oxidized form of coenzyme Q (CoQ-10) percentage in total Q-10 (%CoQ-10) (C) in normal (B) and asphyxiated (J) infants after birth. *, **, *** indicate significant differences ($P < 0.05$, 0.01, and 0.001, respectively) compared to values of normal infants at birth; # and ### indicate significant differences ($P < 0.05$ and 0.001, respectively) compared to the values of infants with asphyxia at birth, as determined by *t*-test. Italic numbers show *P* values between normal infants and infants born with asphyxia at the same age, as determined by *t*-test. *NS* stands for not significant. From [5] with permission.

The percentage of the oxidized form of coenzyme Q-10 (%CoQ-10) in total coenzyme Q-10 was found to be in the range of 25 to 32% during the initial 5 days of life and significantly greater than values generally observed in young healthy adults (4.5%), potentially indicating a condition of oxidative stress at birth. In contrast, %CoQ-10 in infants with asphyxia was found to be significantly elevated (39 to 62%) than values associated with infants having a normal birth (Figure 17.4C). While these results indicate that oxidative stress is more evident in infants with asphyxia, it is worth noting that total levels of coenzyme Q-10 in both groups were equivalent (Figure 17.4B) showing that the measurement of %CoQ-10 offers a true indication of redox status.

17.3.2 Oxidative Stress in Patients with Hepatitis, Cirrhosis, and Hepatoma

Human liver cancer often develops after the onset of chronic hepatitis and the subsequent cirrhosis. Measurement of oxidative stress at each stage is of interest since oxidative stress has been suggested as a causative factor in cancer. However, only a limited number of papers have documented the occurrence of oxidative stress in these liver patients. Therefore, we applied the above described method to plasma obtained from patients with hepatitis, cirrhosis, and hepatoma.[8]

Patients examined in this study were hospitalized at First Department of Medicine in Kyoto Prefectural University of Medicine and were diagnosed with chronic active hepatitis (18 men and 10 women), liver cirrhosis (10 men and 6 women), and hepatocellular carcinoma (15 men and 5 women). All were infected with hepatitis C virus except for 3 patients with hepatitis, 1 with cirrhosis, and 2 with hepatoma who had contracted the hepatitis B virus. The normal subjects consisted of 12 men and 4 women between the ages of 40 and 83 years. Average ages ($\pm$ S.D.) in the four groups were 59.5 $\pm$ 10.7, 60.4 $\pm$ 13.0, 65.7 $\pm$ 7.0, and 57.7 $\pm$ 16.6, respectively, providing no statistical difference.

A significant decrease in plasma ascorbate level was observed in patient groups as compared with normal subjects (data not shown). However, it is also possible that the lowered level of plasma ascorbic acid in these patients was caused by the shortage of ascorbate intake.

We, therefore, adopted a different approach and a newly developed method for the measurement of plasma ubiquinol-10 and ubiquinone-10 was applied. The ratio of ubiquinone-10 to ubiquinol-10 should be one of the most reliable markers of oxidative stress since it is a direct product of redox imbalance. Figure 17.5A shows that patients with chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma had significantly higher content of plasma ubiquinone-10 expressed as %CoQ-10 = ubiquinone-10/(ubiquinone-10 + ubiquinol-10) than normal subjects. These results indicate that oxidative stress is evident after the onset of hepatitis and the subsequent

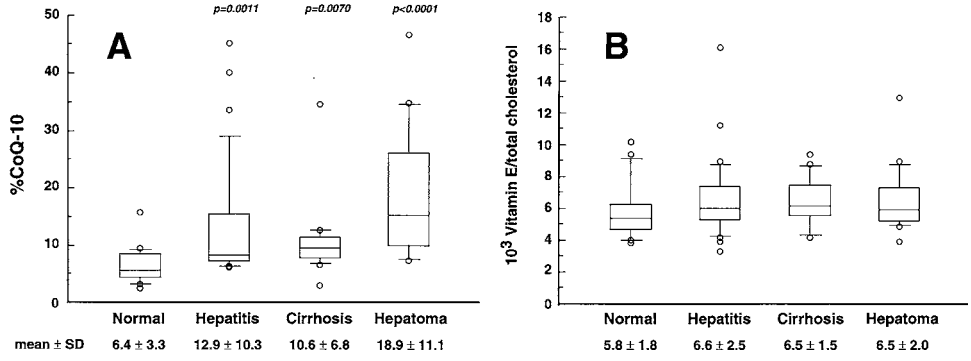

FIGURE 17.5 Box and whisker plots of plasma CoQ-10 percentage (%CoQ-10) in total coenzyme Q-10 (A) and ratio of vitamin E to total cholesterol (B) among normal subjects and patients with chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma (hepatoma). Significant differences compared to normal subjects were analyzed by nonparametric Mann-Whitney test. From [6] with permission.

cirrhosis and liver cancer. A significant increase in the ratio of plasma ubiquinol-9 to ubiquinone-9 was also observed in LEC rat after the onset of hepatitis.[9] On the other hand, vitamin E/total cholesterol ratio remained unchanged among four groups as shown in Figure 17.4B as expected from Figure 17.1.

17.4 CONCLUSION

We described a simple and reproducible method for the detection of ubiquinol-10 and ubiquinone-10 in human plasma. %CoQ-10 in healthy individuals is 4.5%, indicating that plasma coenzyme Q-10 exists mostly in the reduced form. We also demonstrated an increase in oxidative stress in newborn babies and patients with hepatitis, cirrhosis, and hepatoma by measuring %CoQ-10.

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Section 3B

Cardiovascular Pathology

18 Coenzyme Q as a Marker of Oxidative Stress in Coronary Artery Disease

Johan Bernard Ubbink

CONTENTS

18.1 INTRODUCTION: OXIDATIVE STRESS IN CARDIOVASCULAR DISEASE

Lipid peroxidation is a fundamental process in atherogenesis.[1,2] LDL particles are modified by free radical mediated reactions, causing lipid peroxidation and thus oxidative damage to LDL.[1,2] Free radicals are continually formed from metabolic processes occurring in the human body. The most important *in vivo* source of these radical species is univalent, biochemical redox reactions involving oxygen.[3,4]

Since human plasma contains various antioxidants, the extent to which LDL oxidation occurs in the circulation has been thought to be limited.[5,6] Recently, it has been shown that LDL peroxidation starts in the circulation and that patients with certain degenerative diseases may have circulating LDL particles more exposed to oxidative stress than healthy controls.[7] This will be discussed later in this chapter. When LDL enters and becomes trapped in the arterial wall, lipid peroxidation proceeds as chain reactions, which may only be terminated by suitable antioxidants within LDL, such as vitamin E.[1,8] For instance, vitamin E can react directly with a lipid peroxyl radical to render a lipid hydroperoxide and a tocopheroxy radical; both are relatively stable and the chain reaction of continuing lipid peroxyl radical formation is thus terminated.[1,9] However, when LDL is trapped in the vascular wall, mechanisms for vitamin E regeneration are limited, and LDL is presumably rapidly depleted of its vitamin E and other antioxidants content.[10] The antioxidant depleted LDL is subsequently subjected to accelerated lipid peroxidation and fragmentation of lipid peroxides in highly reactive aldehydes,[8,11] which in turn may react with apolipoprotein B[11] to form a modified LDL particle which is recognizable by the scavenger receptors expressed by macrophages.[9,12] Macrophages subsequently internalize oxidized LDL at an enhanced rate, and since the expression of the scavenger receptor is not subject to feedback regulation, this process leads to cellular cholesterol accumulation and foam cell formation.

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Oxidized LDL not only converts macrophages to foam cells, but is also chemotactic for monocytes and T-lympocytes,[13] is known to enhance platelet activation,[14] may stimulate secretion of growth factors,[15] and may affect blood coagulation by perturbing the thrombotic-fibrinolytic equilibrium.[16] It can be concluded that lipid peroxidation contributes significantly to the pathogenesis of atherosclerosis.

18.2 MEASUREMENT OF OXIDATIVE STRESS

Since oxidative stress and the formation of oxidized LDL are fundamental in the process of atherosclerosis, it is not surprising that so many methods have been developed to assess oxidative stress of an individual patient.[17] However, the validity of several of these methods is questionable. For example, circulating malondialdehyde concentrations are widely used to assess oxidative stress, and elevated malondialdehyde levels have even been demonstrated in patients with myocardial infarction,[18] but the significance of malondialdehyde concentration measurement is unclear. It is unlikely to be of a plasma origin, because no circulating lipid hydroperoxides could be demonstrated in human plasma using very sensitive analytical techniques such as HPLC with chemiluminescence detection.[5,6]

Depending on its redox status, coenzyme Q is oxidized and reduced at low potentials.[19] This characteristic of coenzyme Q allows it to fulfill its pivotal role in the electron transport chain.[20] In the circulation, coenzyme Q is mainly carried by lipoproteins,[21] where it is predominantly present in the reduced form of ubiquinol ($CoQ_{10}H_2$). $CoQ_{10}H_2$ in LDL is, however, easily oxidized to ubiquinone (CoQ_{10}). In fact, $CoQ_{10}H_2$ is the first antioxidant to be depleted when LDL is subjected to oxidative stress *in vitro*.[22] As $CoQ_{10}H_2$ is easily oxidized, the $CoQ_{10}H_2/CoQ_{10}$ ratio may be used as a marker of oxidative stress to which circulating LDL has been exposed.[22,23] This ratio may be measured by HPLC using a coulometric detector in an oxidation-reduction-oxidation mode as originally described by Edlund.[19] $CoQ_{10}H_2$ is, however, unstable in whole blood or plasma, and this compound undergoes substantial oxidation within hours after the blood specimen has been obtained.[24] This implies that reliable estimates of the $CoQ_{10}H_2/CoQ_{10}$ could only be made in freshly obtained blood samples subjected to a minimal sample workup before HPLC analysis. This is a severe limitation in using the $CoQ_{10}H_2/CoQ_{10}$ ratio as indicator of LDL exposure to oxidative stress. In our studies on patients with cardiovascular disease, we took particular care to handle samples in such a way that minimal artifactual oxidation could take place.[7] Blood samples obtained from patients or controls were immediately cooled on ice, plasma was separated by low speed centrifugation and immediately thereafter frozen on dry ice. The samples were then transferred to the laboratory on dry ice and stored upon arrival in a $-75\,^{\circ}C$ freezer until analyzed. Upon laboratory analysis, plasma samples were thawed one at a time to avoid artifactual changes of the $CoQ_{10}H_2/CoQ_{10}$ ratio due to oxidation.[7] Subsequently, the plasma sample was extracted with *n*-propanol followed by immediate HPLC analysis.[7,24] The time interval between removal of the sample from the freezer and HPLC analysis of the *n*-propanol extract was usually less than 15 minutes. Using this laborious way of analyses, we obtained $CoQ_{10}H_2/CoQ_{10}$ ratios that were considerably higher than reported by other workers in this field.[24]

18.3 EVIDENCE THAT THE $CoQ_{10}H_2/CoQ_{10}$ RATIO REFLECTS LIPOPROTEIN EXPOSURE TO OXIDATIVE STRESS

Before discussing the measurement of the $CoQ_{10}H_2/CoQ_{10}$ ratio in patients with cardiovascular disease, it is imperative to show that this ratio is indeed reflective of oxidative stress. This was done in a recent study, where we examined the *in vitro* oxidation of plasma coenzyme Q_{10} when subjected to various concentrations of Cu^{2+}. To prevent complex formation of Cu^{2+} with EDTA, we used serum for this experiment.[7] Figure 18.1 shows that low concentrations of Cu^{2+} (5 μmol/L) caused a small increase in the CoQ_{10} concentration, but this had a substantial effect on the $CoQ_{10}H_2/CoQ_{10}$ ratio. After 20 minutes of incubation with 5 μmol/L $CuSO_4$, the $CoQ_{10}H_2/CoQ_{10}$

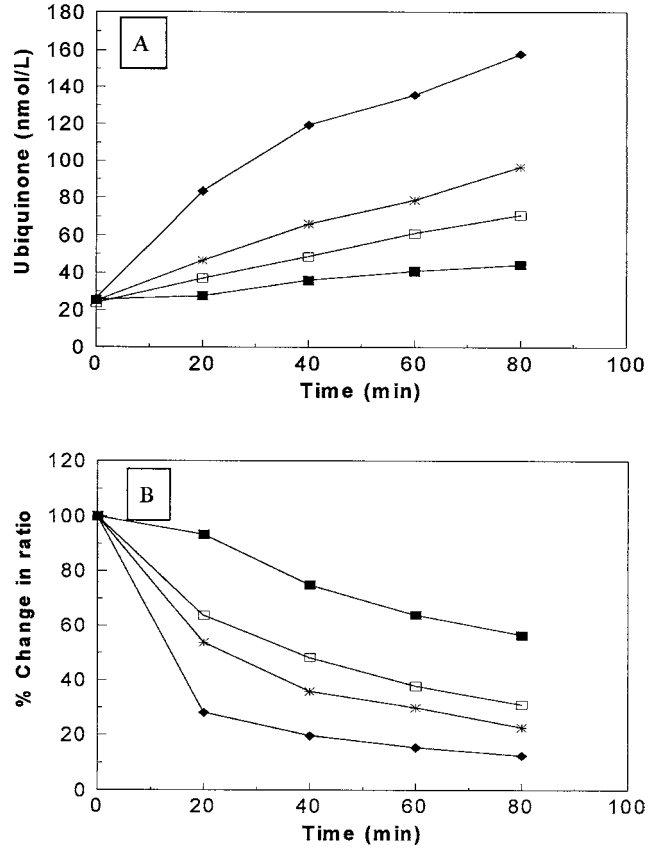

FIGURE 18.1 A. Increase in serum ubiquinone (CoQ_{10}) when serum, preincubated at 30°C, was subjected to (■) water (control); (□) 5 μmol/L $CuSO_4$; (∗) 10 μmol/L $CuSO_4$; and (◆) 100 μmol/L $CuSO_4$. B. Percentage decline in the ubiquinol/ubiquinone ($CoQ_{10}H_2/CoQ_{10}$) ratio when serum was subjected to oxidation by treatment with various concentrations of $CuSO_4$: (■) water (control); (□) 5 μmol/L $CuSO_4$; (∗) 10 μmol/L $CuSO_4$; and (◆) 100 μmol/L $CuSO_4$. Adapted with permission from the publisher from [7].

ratio decreased to 36.2%, compared with a 6.7% decrease of the control sample. It is remarkable that the $CoQ_{10}H_2/CoQ_{10}$ ratio changed so rapidly in whole serum, which contains various antioxidants at relatively high concentrations. These results therefore indicate that the $CoQ_{10}H_2/CoQ_{10}$ ratio is sensitive to oxidative stress and may be used to estimate the exposure of circulating lipoproteins to free radical reactions. An altered $CoQ_{10}H_2/CoQ_{10}$ ratio may be the first sign of lipoprotein exposure to oxidative stress, because $CoQ_{10}H_2$ is the first antioxidant to be depleted when LDL is exposed to free radical production.[22,23] These results therefore support the concept that the $CoQ_{10}H_2/CoQ_{10}$ ratio could be used to evaluate the oxidative stress to which circulating lipoproteins are exposed in patients with cardiovascular disease.

18.4 $CoQ_{10}H2/CoQ_{10}$ RATIO IN PATIENTS WITH CARDIOVASCULAR DISEASE

Coenzyme Q_{10} extracted from both atherosclerotic plaque and normal segments from human iliac or carotid arteries was detected only in the oxidized form (CoQ_{10}). This shows that the bulk of $CoQ_{10}H_2$ oxidation occurs when LDL is trapped in the intimal space.[25] But what is the situation with coenzyme Q_{10} contained in LDL?

TABLE 18.1
Coenzyme Q_{10} Parameters and Antioxidants Measured in Plasma of Patients with Angiographically Proven Cardiovascular Disease and Controls

Parameter	Cardiovascular Disease (n = 40) Mean (SD)	Controls (n =100) Mean (SD)	Statistics p-value
Age (years)	52.6(9.9)	40.8(9.6)	$p < 0.001$
$CoQ_{10}H_2$ (nmol/L)	1137.0(452.3)	1145.1(360.9)	N.S.
CoQ_{10} (nmol/L)	46.4(23.6)	40.3(15.0)	N.S.
$CoQ_{10}H_2/CoQ_{10}$ ratio	26.5(7.5)	30.2(8.8)	$p = 0.02$
Cholesterol (mmol/L)	5.4(1.2)	5.5(1.2)	N.S.
Vitamin E (μmol/L)	32.8(8.9)	29.2(8.4)	$p = 0.025$

Source: Reproduced with permission of the publisher from reference [7].

We studied the $CoQ_{10}H_2/CoQ_{10}$ ratio in forty consecutive male patients (aged 26 to 66) years, who were admitted to the Pretoria Heart Hospital with angiographically proven cardiovascular disease.[7] Of these patients, 29 had previously suffered myocardial infarction or had undergone coronary artery bypass surgery. The remaining 11 patients had greater than 50% stenosis of at least one of the main coronary arteries. Nine patients of the total group were treated with lipid lowering agents, eight of which were on HMG Co-A reductase inhibitors. The control group consisted of 100 apparently healthy male subjects (aged 24 to 62) with no history of coronary artery disease.

In the patient group, arterial blood samples (5 ml) with EDTA as anticoagulant, and (5 ml) clotted blood, were obtained using an arterial sheath placed in the femoral artery with the Seldinger technique under local anesthetic prior to coronary angiography. In controls, blood samples were collected cubitally with venous puncture. The blood samples were processed at the "point-of-care" as described above and were then transferred to the laboratory on dry ice and stored upon arrival in a -75°C freezer until analysed for $CoQ_{10}H_2$, CoQ_{10}, and vitamin E.

The results are summarized in Table 18.1. Cardiovascular disease patients had significantly lower $CoQ_{10}H_2/CoQ_{10}$ ratios compared with controls. As age was found to be a significant cofactor for the $CoQ_{10}H_2/CoQ_{10}$ ratio, this ratio was corrected for age and the above-mentioned differences became more pronounced; the respective adjusted mean ratios were 25.2 and 30.7 ($p < 0.001$). Within group comparisons in the patient group showed that treatment with HMG Co-A reductase inhibitors or previous cardiovascular events did not bias the findings. It is remarkable that the plasma vitamin E concentrations in this study were significantly higher in cardiovascular disease patients compared with controls. While no apparent reason for this difference could be found, this observation at least indicates that the altered $CoQ_{10}H_2/CoQ_{10}$ ratio in patients cannot be explained by differences in plasma vitamin E (main lipid-soluble antioxidant) content.

Our results indicate that CAD patients have significantly lower $CoQ_{10}H_2/CoQ_{10}$ ratios compared with controls, indicating that lipoproteins in general, and LDL in particular, were exposed to higher levels of oxidative stress in the patient group. Furthermore, the results suggest that oxidative LDL modification starts in the circulation, however, the impact that this process may have on vascular disease progression is still unclear. It is possible that the process may affect apo B[26] resulting in altered (increased atherogenic) properties of circulating LDL.

Little work has been done to confirm the above-mentioned findings that LDL in the circulation from cardiovascular disease patients is exposed to higher levels of oxidative stress as characterized by an altered $CoQ_{10}H_2/CoQ_{10}$ ratio. Cleary and coworkers[27] studied 32 patients with confirmed, severe coronary artery disease and compared them to 24 age-matched controls. In agreement with our results, these authors found lower values of total coenzyme Q_{10} and a lower coenzyme Q redox status (defined as the percentage of $CoQ_{10}H_2$ to the total coenzyme Q) in patients compared with controls, while plasma vitamin E concentrations were higher in patients. Neither of these differences were, however, statistically significant. It is possible that the study of Cleary et al. lacked the statistical power to demonstrate statistically significant

differences in the $CoQ_{10}H_2/CoQ_{10}$ ratio between cardiovascular disease patients and controls. On the other hand, it should be noted that $CoQ_{10}H_2/CoQ_{10}$ ratios measured by Cleary and coworkers are considerably lower when compared to our results.[27] Using the mean values for $CoQ_{10}H_2$ and CoQ_{10} published by Cleary et al., the $CoQ_{10}H_2/CoQ_{10}$ ratios for patients and controls were 3.32 and 3.96, respectively. These ratios are at least sixfold lower than those reported by Lagendijk et al.[7] Although the authors were very careful in trying to avoid $CoQ_{10}H_2$ oxidation during the extraction procedure, it is possible that their lower $CoQ_{10}H_2/CoQ_{10}$ ratios reflect $CoQ_{10}H_2$ autoxidation that occurred during the extraction procedure. We found that the more complex the extraction procedure, the more likely it is for $CoQ_{10}H_2$ autoxidation to occur, no matter how meticulously the analyses are performed. It is for this reason that the analytical method utilized in our studies uses minimal sample clean up procedures.[24] In absence of appropriate cleanup procedures, the coulometric detectors may be subjected to sample overload. This was overcome in our procedure by installation of a 2-way valve between the analytical column and the first coulometric cell, i.e., the conditioning cell.[24] Using this valve, the column eluant of the first 2 minutes of the HPLC run was directed past the detectors to a waste container, thus protecting the coulometric cells from the bulk of interfering material that eluted early from the analytical column.[24] This innovation made it possible to inject isopropanol extracts from serum directly onto the HPLC column, without causing overloads at the coulometric detectors. It is suggested that this procedure minimizes $CoQ_{10}H_2$ autoxidation, explaining the substantial higher $CoQ_{10}H_2/CoQ_{10}$ ratios in our study compared to others.

A recent study of De Rijke et al.[28] demonstrates how difficult it is to interpret redox status measurements of coenzyme Q_{10} . These authors found that subjects with small, dense LDL, which is prevalent in patients with coronary heart disease, had a mean (SD) $CoQ_{10}H_2/CoQ_{10}$ ratio in isolated LDL of 1.9 (1.0), which was significantly lower than the observed ratio of 3.2 (1.0) in participants with larger, more buoyant LDL. The authors conclude that small, dense LDL particles are more prone to oxidative modification. This seems indeed true, but this does not imply that small dense LDL is more prone to oxidative stress *in vivo.* It is quite possible that small dense LDL is more susceptible to artifactual oxidation during the analytical preparation procedure due to its small size. The possibility that artifactual oxidation may have contributed to the observations of De Rijke et al., is strengthened by the low $CoQ_{10}H_2/CoQ_{10}$ ratios reported by these authors.[28]

18.5 CONCLUDING REMARKS

The use of the $CoQ_{10}H_2/CoQ_{10}$ ratio as an indicator of *in vivo* oxidative stress to which LDL is exposed is an analytically challenging concept. It is not simple to determine the $CoQ_{10}H_2/CoQ_{10}$ ratio from a plasma sample. Current methodology relies on HPLC with coulometric detection to quantify both $CoQ_{10}H_2$ and CoQ_{10} in a single run. This presumably explains why the $CoQ_{10}H_2/CoQ_{10}$ ratio has rarely been used in assessing the possible role of oxidative stress in cardiovascular disease. A further complicating factor is the susceptibility of $CoQ_{10}H_2$ to oxidation during the analytical quantification procedure. Artifactual oxidation may have contributed to contradictory results reported by different groups in this field.

For the future use of $CoQ_{10}H_2/CoQ_{10}$ ratios in the assessment of oxidative stress, it is imperative that the measurement of $CoQ_{10}H_2$ and CoQ_{10} be standardized. This is a tall order, because it implies that reference material should be available and that a reference method should be defined. However, this is the only way forward and will solve the discrepancies between different laboratories on *in vivo* LDL exposure to oxidative stress in cardiovascular diseases.

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19 Role of Coenzyme Q_{10} in Myocardial Tolerance to Ischemia and Reperfusion

Hajime Otani and Dipak K. Das

CONTENTS

19.1 INTRODUCTION

Heart failure is the leading cause of death and debilitating conditions in industrialized countries. Congenital heart anomaly, valve dysfunction, coronary artery disease, hypertension, myocarditis, metabolic disorder, and idiopathic cardiomyopathy are known to deteriorate to heart failure. Although the mechanism of heart failure differs among the underlying heart diseases, there are common features of heart failure. Heart failure is characterized by impairment of the contractile function of cardiac muscle. The pump function of cardiac muscle is determined by the number of functional cardiomyocytes and the contactility of each cardiomyocyte. When approximately 40% of contractile mass of the left ventricle is lost by acute myocardial infarction, the remaining left ventricular muscle cannot compensate for reduced cardiac performance, leading to heart failure. Cardiomyocyte contractility is determined by the velocity and the strength of contraction and relaxation of myofilaments. A number of factors are involved in this regulation and most of them are dependent on energy. Cross-bridge cycling between actin and myosin consumes the largest part of energy for cardiomyocyte contraction. Regulation of cytosolic Ca^{2+} level is also important in generating contractility of myofilaments. The intracellular Ca^{2+} level in each contraction and relaxation cycle is mainly regulated by Ca^{2+}-ATPases present in the sarcoplasmic reticulum and the sarcolemma. Thus, energy deprivation of cardiac myocytes directly affects contractile function. Depletion of myocardial high energy phosphates occurs as a result of deficient production of ATP by mitochondria or excess utilization of energy by ATPases. Therefore, stimulating ATP synthesis by mitochondria represents a promising approach for treatment of patients with heart failure.

19.2 COQ AS A CARDIOPROTECTIVE DRUG

Coenzyme Q_{10} (CoQ) has long been utilized as a cardioprotective drug treating myocardial ischemic heart disease, heart failure, and cardiotoxic chemical intoxication. As a member of the mitochondrial electron transfer chain, CoQ is directly involved in energy transduction and aerobic ATP production; it

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transports electrons in the respiratory chain and couples the respiratory chain to oxidative phosphorylation.[1,2] In addition, CoQ is a powerful antioxidant not only within the mitochondria but also in other organelle membranes containing CoQ.[3] It is now apparent that reactive oxygen species (ROS) are a common mediator of cytotoxic stress. The biochemical mechanisms underlying the toxicity of ROS are their ability to peroxidize membrane phospholipids with unsaturated free fatty acid and interaction with certain sulfhydryl proteins essential for maintaining normal cell function. The net result of free radical-induced damage appears to be altered membrane function and structure. Eventually, the altered handling of ionic gradients results in intracellular calcium overload leading to activation of calcium-dependent degradation enzymes such as calcium-activated neutral proteases and phospholipases, wasting of energy by activating calcium-dependent ATPase, and mitochondrial dysfunction due to energy-dependent uptake of calcium by mitochondria. Accumulation of calcium in the mitochondrial matrix above the critical level results in the activation of cell death cascade as will be discussed later.

The rationale for employing ROS scavengers in the face of ischemia and reperfusion is based on the fact that ischemia and reperfusion increase free radical generation and that an antioxidative defense system is compromised during these periods. Several animal studies support the theory of ROS-induced myocardial damage during ischemia and reperfusion. Myocardial reperfusion has been shown to increase ROS generation.[4–6] On the other hand, the activity of superoxide dismutase and glutathione peroxidase is reduced and cellular glutathione is depleted during myocardial ischemia and reperfusion.[7,8] Pretreatment of animals with ROS scavengers has been demonstrated to reduce myocardial injury and improve cardiac function during reperfusion.[9–11] Likewise, the studies examining CoQ as a therapeutic agent indicate that its major action in protecting the heart from reperfusion damage is primarily derived from antioxidation. The feasibility of CoQ treatment has been supported by several studies. The level of endogenous CoQ decreases during reperfusion, and administration of CoQ increased mitochondrial CoQ level and inhibited its reduction during reperfusion.[12,13] Biosynthesis of CoQ after reperfusion is impaired especially in aged animals.[14] Finally, mitochondrial CoQ content is decreased after simulated reperfusion associated with free radical generation.[15]

Myocardial protection by exogenous CoQ was first reported by Nayler.[16] She demonstrated that rat hearts pretreated with CoQ had significantly less depletion of ATP and less severe ultrastructural changes compared to controls after postischemic reperfusion. Since then, numerous animal studies have been performed using CoQ as a cardioprotectant and most of them have proven that exogenous CoQ is useful in myocardial protection. The beneficial effects of CoQ on myocardial energy metabolism have been most convincingly demonstrated in global ischemia models. CoQ treatment was capable of increasing myocardial high energy phosphate compounds following reperfusion [17,18] and improving left ventricular function.[19] Animal studies of acute myocardual infarction also have shown improvement of left ventricular function and inhibition of ultrastructural deterioration after acute occlusion of coronary arteries by preischemic intravenous administration of CoQ in rats and dogs.[20,21] However, it has been shown that acute administration of CoQ failed to reduce infarct size after acute coronary artery occlusion and reperfusion in rabbits.[22] The reason for this apparent discrepancy is unknown, but may be related to differences in species and in parameters of myocardial protection. In contrast, Ferrara and coworkers reported that after 4 weeks of dietary supplementation with CoQ_{10}, tissue concentration of CoQ_{10} was elevated by 22%, and oxidative stress was significantly suppressed.[23] Morita and coworkers showed that administration of CoQ_{10} before the onset of reoxygenation on cardiopulmonary bypass reduced oxygen-mediated myocardial injury and attenuated myocardial injury after cardiopulmonary bypass in pigs.[24] In a recent study, a group of pigs were fed coenzyme Q_{10} supplements with their regular diets for 30 days while another group of pigs were fed a regular diet supplemented with a placebo for the same time period and served as controls. At the end of 30 days, isolated in situ pig hearts were prepared and hearts perfused with a cardiopulmonary pump system. Each heart was subjected to 15 minutes of regional ischemia by snaring LAD followed by 60 minutes of hypothermic cardioplegic global ischemia and 60 minutes of normothermic reperfusion. Contractile function was evaluated by measuring left ventricular developed pressure (LVDP) at preischemic baseline and during

reperfusion. Blood perfusate was collected at the preischemic baseline and during reperfusion to estimate creatine kinase (CK) and malonaldehyde (MDA) contents. At the end of the experiments, myocardial infarct size was measured by TTC staining methods. Separate groups of pigs (CoQ_{10}-fed and unfed) were used to assess CoQ_{10} content. The CoQ_{10} fed group revealed higher content of CoQ_{10} (21.5 $\pm$ 0.7 vs. 28.0 $\pm$ 0.5 μg/g heart) indicating bioavailability of CoQ_{10} in heart. Postischemic left ventricular contractile function was better recovered in the CoQ_{10} group as compared with the control group of pigs. For example, at the end of 2 hours of reperfusion, developed pressure (DP) (92 $\pm$ 3.9 vs. 131 $\pm$ 4.2 mmHg) and maximum first derivative of DP ($LV_{maax}dp/dt$) (1110 $\pm$ 98 vs. 1976 $\pm$ 85 mmHg/sec) were higher for the hearts of CoQ_{10}-fed pigs. CoQ_{10}-fed pigs revealed smaller myocardial infarctions and lesser CK release from the coronary effluent compared to those for the non-CoQ_{10}-fed animals. The CoQ_{10} group of pigs demonstrated lesser amounts of MDA in the coronary effluent and a higher content of antioxidant reserve in the heart. The results of this study demonstrated that nutritional supplementation of CoQ_{10} could render the hearts resistant to ischemic reperfusion injury probably by reducing the oxidative stress.

The effects of CoQ on patients with ischemic heart disease have been investigated. Hiasa et al.[23] evaluated exercise tolerance in a placebo-controlled trial utilizing intravenous administration of CoQ 1.5 mg/kg once daily for 7 days versus placebo in 18 patients with chronic stable angina. The mean exercise time in the CoQ group at day 7 had significantly increased compared to placebo treatment, suggesting that CoQ treatment induced tolerance to myocardial ischemia. Randomized, double-blind placebo-controlled trials of oral administration of CoQ have confirmed the effectiveness of CoQ in improving anginal episodes, arrhythmias, and left ventricular function in patients with acute myocardial infarction.[24] The potential benefit of long-term oral administration of CoQ has emerged from the clinical trial for patients with chronic heart failure. CoQ is deficient in patients with congestive heart failure[25] and supplimentation of CoQ benefits such patients.[26] The efficacy of long-term CoQ treatment on cardiac function and myocardial energy metabolism has been confirmed experimentally in rats with chronic heart failure.[27] CoQ has been employed in treatment for adriamycin cardiotoxicity. Many years ago, adriamycin, an anthoracycline, and mixed quinoid and hydroquinoid compounds were shown to have inhibitory effects on CoQ enzyme systems[28] and several experimental studies demonstrated that exogenous CoQ prevented adriamycin-induced myocardial damage.[29,30] CoQ has also been employed in attempts to improve postischemic cardiac function in open heart surgery. Either oral administration for 7 days before surgery or intravenous administration 30 minutes before cardiopulmonary bypass was shown to be effective in mitigating postoperative pump failure.[31,32] Another study,[33] however, failed to demonstrate myocardial protection during cardiac operations by short-term oral supplementation with CoQ. The differences of effectiveness by exogenous CoQ may in part be due to its hydrophobic nature, which prevents CoQ from gaining access to intracellular organelles where CoQ exerts cytoprotective action. Perhaps optimal tissue distribution of CoQ requires several days by oral administration, but can be shortened by intravenous treatment of liposomal form of CoQ. Cardioprotective effects of CoQ are not confined to cardiomyocytes, but are also beneficial in improving coronary endothelial function.[34] Protection of both cardiomyocytes and endothelial cells from reperfusion injury could synergistically enhance the recovery of myocardial function during reperfusion. In summary, although there is some controversy on the efficacy of CoQ in treating cardiovascular diseases, the prevailing opinion suggests that CoQ may have a potential role in protecting myocardium from energy depletion and ROS overproducing events.

19.3 HYPOTHETICAL MECHANISM OF MYOCARDIAL ACQUISITION OF TOLERANCE TO ISCHEMIA AND REPERFUSION BY COQ

One of the important mechanisms by which CoQ exerts cardioprotection is related to its role as a mobile electron carrier in the mitochondrial electron-transport process of respiration and coupled

phosphorylation.[35] The ability of CoQ to afford myocardial protection is also attributed to its antioxidant and membrane stabilizing properties. However, there are controversial issues as to whether CoQ acts as an antioxidant or prooxidant.[36,37] O_2^- can stem from a divergent single electron transfer from redox cycling ubisemiquinone. It has been demonstrated that exogenously added CoQ enhances O_2^- generation in isolated complex I (NADH-CoQ reductase) and III (CoQ-cytochrome *c* reductase).[38] Other lines of evidence, which also support redox cycling of CoQ as an alternative site of direct oxygen interaction during respiration, were derived from the experiments showing that H_2O_2 release from decomposing O_2^- was inhibited after removal of CoQ from mitochondria, but was reestablished after reincorporation of added CoQ.[39] Myxothiazol, which prevents the existence of ubisemiquinone at its outer binding center to the bc_1 complex, inhibited mitochondrial O_2^- formation.[40] Arguments against the role of CoQ as the source of O_2^- have been provided by Nohl and Stolze[41] who reported that O_2^- formation did not occur through redox cycling of CoQ in a water-free nonpolar reaction system that resembles the lipophilic character of the inner mitochondrial membrane, but became significant when the membrane was permeable to protons by toluene pretreatment. This observation suggests that CoQ may not play a major role in O_2^- generation in intact mitochondria, but may represent an important source of O_2^- generation under a pathological condition in which the inner mitochondrial membrane is protonated by certain pathological events such as ischemia/reperfusion.[42]

The antioxidant role of CoQ has been investigated in more detail. The ability of reduced CoQ to interfere with the formation of free radical-induced chemical changes in liposomes, lipid emulsions, and other purely chemical systems has been confirmed, extended, and reported in a significant number of publications. Above all, the main function of CoQ as an antioxidant appears to inhibit lipid peroxidation, since localization of CoQ in the hydrophobic region of the membrane phospholipid bilayer is a particularly favorable position to inhibit lipid peroxidation and oxidative damage of proteins associated with the membranes. Although the exact nature of such dual regulation of CoQ in generating and scavenging O_2^- has not been elucidated, it is assumed that both oxidative and antioxidative mechanisms play an important role in acquisition of tolerance to ischemia and reperfusion by CoQ.

It is increasingly clear that redox signaling plays an important role in cell survival and tolerance under various noxious stimuli. ROS possess a wide variety of functions in cell physiology and biochemistry. Burst generation of ROS is dangerous for survival of cells. However, aerobic organisms are known to take advantage of a small amount of ROS as a sensor to stimulate signal transduction pathways that feed back to protect against lethal injury as a result of massive generation of ROS. Recent studies have raised the hypothesis that a brief period of hypoxia could generate ROS signaling in cardiac myocytes that provoke adaptive mechanisms to protect against chronic deprivation of oxygen, a phenomenon called ischemic preconditioning.[43–45] These results also suggest that mitochondria are the source of ROS generation during preconditioning. Prooxidant function of CoQ may be involved in the generation of this ROS signaling and activation of cellular defense systems against massive generation of ROS. A variety of mitochondrial poisons are well known to stimulate ROS generation by a mechanism that involves inhibition of electron transfer, accumulation of reducing equivalents in the middle portion of the electron transfer chain, and direct one-electron transfer to oxygen to produce O_2^-.[46] This switch appears to occur principally at the level of CoQ. It has been demonstrated that loss of cytochrome *c* plays a triggering role in O_2^- generation by mitochondria during apoptotic stimuli.[47] The release of cytochrome *c* from the mitochondria to the cytosol is a key event in activating an apoptotic process that involves cysteine proteases and caspases. The loss of cytochrome *c* from the mitochondrial electron transfer chain interrupts electron flow at the level distal to CoQ-cytochrome *c* reductase leading to enhanced generation of O_2^-. The working hypothesis is derived from the loss of cytochrome *c* as a trigger for both apoptosis and ROS signaling, which stimulates the cellular defense system against apoptosis.

Intracellular Ca^{2+} overload is a unifying feature of cell injury by a variety of noxious stimuli. Myocardial ischemia and reperfusion cause an increase in cytosolic Ca^{2+}. There are several mechanisms by which cytosolic Ca^{2+} increases during ischemia and reperfusion. However, there is a

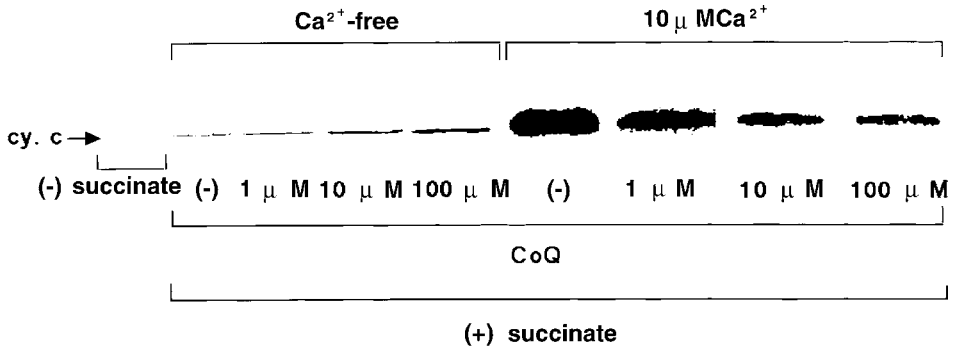

FIGURE 19.1 Effects of coenzyme Q10 (CoQ) on cytochrome *c* release from the isolated rat heart mitochondria. Mitochondria were suspended in the buffer containing 180 mM KCl, 5 mM MOPS, 2 mM EGTA, pH 7.25. Cytochrome *c* (cy. *c*) release from the mitochondria was measured after 30 minutes incubation in the absence or presence of 5 mM succinate at 25°C. Cy. *c* released into the supernatant of the mitochondrial suspension was detected by western blot analysis. Cy. *c* release was not found in nonrespiring mitochondria without succinate. However, small but appreciable amounts of cy. *c* release were always observed in the mitochondria in the presence of succinate even under a Ca^{2+}-free condition. Preincubation for 60 minutes with CoQ at concentrations ranging between 1 and 100 μM increased cy. *c* release in a dose-dependent manner. When a free Ca^{2+} concentration of the buffer was adjusted to 10 μM, there was a large amount of cy. *c* released into the supernatant of the mitochondrial suspension. CoQ inhibited this cy. *c* release in a dose-dependent manner.

general agreement that Na^+/H^+ exchange plays a pivotal role in intracellular Ca^{2+} overload during reperfusion.[48] Na^+/H^+ exchange is activated during reperfusion associated with recovery from intreacellular acidosis that results in accumulation of intracellular Na^+. Intracellular Na^+ is then extruded from the cells via Na^+/H^+ exchange leading to an influx of Ca^{2+}. Increased cytosolic Ca^{2+} is taken up by energized mitochondria, which in turn induces opening of the mitochondrial permeability transition pore, a mega channel through which solutes with molecular masses </= 1500 Da enter mitochondria. This step is crucial, not only for necrosis but also for apoptosis. The Ca^{2+}-induced opening of the permeability transition pore promotes extrusion of accumulated Ca^{2+} from the mitochondria,[49,50] thus producing a futile cycle of Ca^{2+} uptake and release, which leads to ATP depletion and necrosis. Opening of the permeability transition pore also induces release of cytochrome *c* and apoptosis-inducing factor into the cytosol.[51,52] These mitochondria-derived proapoptotic molecules then activate the cysteine protease cascade responsible for apoptosis.

Since CoQ appears to interfere with the process of cardiomyocyte cell death, we have asked if CoQ exerts myocardial protection by affecting Ca^{2+}-induced cytochrome *c* release. We have examined this possibility using isolated rat heart mitochondria. Mitochondria were isolated from 8 to 10-week-old Sprague-Dawley rat heart with a conventional method. As shown in Figure 19.1, there was respiration-dependent release of cytochrome c from the mitochondria. State 2 respiration, which did not increase O_2 consumption measured by an O_2 electrode, did not induce cytochrome *c* release, while state 4 respiration induced by addition of succinate, provoked a small but an appreciable amount of cytochrome *c* release. Interestingly, preincubation with CoQ at concentrations ranging from 1 to 100 mol/L for 60 minutes increased cytochrome c release at a physiological Ca^{2+} concentration. However, a large amount of cytochrome *c* release induced under a high Ca^{2+} condition was inhibited by preincubation with CoQ at concentrations above 10 μmol/L. Thus, CoQ produced a dual effect on cytochrome *c* release from the mitochondria. CoQ promotes a small amount of cytochrome *c* release when extramitochondrial Ca^{2+} concentration is physiological, whereas it suppresses a large amount of cytochrome *c* release when extramitochondrial Ca^{2+} concentration is increased to a pathological level. Assuming that cytochrome *c* release is correlated

with ROS generation, it is hypothesized that CoQ plays a triggering role in ROS signaling for developing a defense system against ROS during low-grade oxidative stress while preventing ROS-induced damage by inhibiting lipid peroxidation upon a catastrophic increase in ROS production. These indirect and direct mechanisms of protection against oxidative stress would render myocardium to be highly tolerant to ischemia and reperfusion.

The most important candidate for CoQ therapy may be the aged population. It has been shown that myocardial CoQ content drops drastically in elderly rats.[53] The age-dependent difference in CoQ content may explain reduced tolerance to ischemia and reperfusion in elderly patients. It has been reported that CoQ content of isolated human atrial trabeculae from those over 70 years of age is significantly lower compared to that from those under 70, and that pretreatment of the trabeculae with CoQ overcomes the reduced capacity of aged trabeculae to recover contractile function after ischemia compared to younger tissue.[54] Since senescent human myocardium is known to be less tolerant of ischemia and reperfusion, decreased CoQ content in aged human myocardium may contribute to the reduced recovery of cardiac performance in aged populations suffering from acute myocardial infarction and undergoing cardiac operations.

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20 The Role of Coenzyme Q in Controlling the Endothelial Function and Arterial Tone

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CONTENTS

20.1 INTRODUCTION

The control of blood pressure is one of the most complicated multiorgan functions in humans. The central nervous system (CNS) regulates blood pressure by adjusting the heart rate and contractility as well as peripheral resistance. This occurs mainly via the sympathetic and parasympathetic pathways of the autonomic nervous system, but neuroendocrine pathways, such as the hypothalamo-pituitary axis, are also involved.[1] The renal perfusion pressure is involved in blood pressure regulation via the renin-angiotensin system (RAS).[2,3] During the past two decades, it has become evident that the control of arterial tone is also crucial in the regulation of blood pressure. Under normal conditions, resistance arteries remain in a contracted state, from which they can dilate or constrict depending on neuronal and humoral stimuli and local vascular control mechanisms. An adequate degree of contraction is crucial for the maintenance of arterial blood pressure. Thus, resistance arteries play an important role in the regulation of arterial blood pressure.[4]

20.2 LOCAL CONTROL OF ARTERIAL TONE

Arterial tone is locally controlled by a fine interplay between endothelial cells lining these arteries and smooth muscle cells surrounding them. In response to neuronal, humoral, and physical stimuli,

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endothelial cells are known to release a variety of relaxing and contracting factors that regulate the underlying smooth muscle.[5] According to present knowledge, the most important relaxing factors are thought be nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF).[5] In addition to dilating substances, the endothelium can release various endothelium-derived contractile factors (EDCF) such as endothelin-1, angiotensin II, thromboxane A_2, and prostaglandin H_2 (PGH_2). However, the contribution of these factors to blood pressure regulation remains as yet undefined.[6]

The above-mentioned endothelium-derived relaxing and contracting factors exert their actions in smooth muscle cells either by binding to a specific receptor on the smooth muscle membrane and a consequent activation of a second-messenger-mediated pathway, or by direct activation of second-messenger pathways. PGI_2 binds to a membrane receptor on smooth muscle, which activates the enzyme adenylate cyclase, causing an increase in the intracellular concentration of cyclic adenosine 3',5'-monophosphate (cAMP).[7] NO, as a small molecule, diffuses into smooth muscle cells and directly activates guanylate cyclase, which leads to enhanced formation of cyclic guanosine 3',5'-monophosphate (cGMP).[8] The end result of the increase in both cAMP and cGMP is a reduction in the amount of free intracellular Ca^{2+} available for contraction and thus a relaxation of smooth muscle cells.[8] EDHF is thought to bind directly to K^+ channels on the smooth muscle cell membrane and result in a hyperpolarization of these cells. Further, this reduces the influx of Ca^{2+} ions through voltage-dependent Ca^{2+} channels, thus resulting in a relaxation of smooth muscle cells.[8]

20.3 DETERIORATION OF ARTERIAL FUNCTION WITH AGING

Aging is associated with changes in the structure and function of the arteries.[9] The function of both endothelium and vascular smooth muscle cells is usually affected with age.

The effect of age on endothelium-mediated responses varies with species and vascular bed.[10] In general, there is a tendency toward a reduction in endothelium-dependent relaxations.[9] In contrast, relaxation of aged vessels in response to nitrovasodilators is essentially well maintained[11,12] although contradictory results have also been published.[13] The reduction in endothelium-dependent vasodilations of aged arteries may be contributed to by a decrease in the synthesis of NO. It has also been suggested that the reduction could be due to an inhibition of NO access to smooth muscle cells by the thickening of endothelial and smooth muscle layers in aging.[9]

Changes also take place with aging in the endothelium-independent vasodilations. The relaxation caused by the β-adrenoceptor agonist isoprenaline is especially reduced during aging.[9,14] A reduction in β-adrenergic responses during aging is a very common phenomenon.[15] The reduction in β-receptor-mediated responses in the arteries reduces the efficacy of the sympathetic system in the control of the cardiovacular system.[9] There are contradictory results regarding whether the reduction in the response to β-agonist is due to a decrease in the cellular density of β-receptors and in the affinity for β-agonist binding,[16] a decrease only in β-density without a change in their affinity,[17] or to other factors.[14,18] However, all mechanisms that participate in cellular desensitization of β-adrenergic stimulation with aging lead to the same result, i.e., insufficient production of the second messenger cAMP following β-stimulation.[14,18]

20.4 COENZYME Q AND ARTERIAL FUNCTION

The role of coenzyme Q in the regulation of arterial function has been recognized only recently by Yokoyama and colleagues.[19] They studied the effect of exogenous coenzyme Q on arterial function. In their study, rats were pretreated with 10 mg/kg intraperitoneal and 20 mg/kg intramuscular coenzyme Q_{10} boluses 24 and 2 hours before the experiment with isolated perfused hearts. Global normothermic ischemia led to a deterioration of coronary vasorelaxation induced by either bradykinin, an endothelium-dependent vasodilator, or sodium nitroprusside, an endothelium-independent NO donor. Also, infusion with hydrogen peroxide (H_2O_2) as a direct source of oxygen-derived

radicals led to a similar impairment of bradykinin-induced vasorelaxation. The impairment of endothelium-independent vasorelaxation was not as profound as that of endothelium-dependent vasorelaxation, suggesting that the endothelium is particularly vulnerable to free radical burst during the reperfusion period. In this study, pretreatement with coenzyme Q_{10} protected endothelium-dependent but not endothelium-independent vasodilatory responses from ischemia/reperfusion- and H_2O_2-induced injuries. Furthermore, the free radical burst during the reperfusion period, measured by lucigenin-enhanced chemiluminescence, was significantly reduced by coenzyme Q_{10} pretreatment, and thus the authors suggest that coenzyme Q_{10} protects the endothelium via a direct antioxidant mechanism.

20.5 MATERIALS AND METHODS OF OUR OWN STUDIES

20.5.1 Mesenteric Artery Function after Long-Term Supplementation of Coenzyme Q

A total of 18 senescent (age 16 months) and 9 young (age 2 months) male Wistar rats were used to study mesenteric arterial function. Prior to the experiment, all animals had been maintained on a standard diet. From the age of 16 months, 9 rats were supplemented with 10 mg/kg/day coenzyme Q_{10} for 8 weeks while the rest of the senescent rats, as well as all young rats, were kept on standard diet. After the 8-week supplementation, the animals were decapitated and blood samples collected from the decapitation line. The superior mesenteric arteries were carefully excised and cleaned of adherent connective tissue and transferred to an organ bath to study the function of the mesenteric artery (see below).

20.5.2 Coenzyme Q Measurements in Plasma

The coenzyme Q concentrations in the plasma samples were measured by an independent laboratory (MILA laboratories, Helsinki, Finland) according to Okamoto et al.[20] with some modifications. The serum samples were extracted with *n*-propanol and coenzyme Q_7 was added as an internal standard. The coenzymes were reduced with $NaBH_4$ prior to HPLC, employing a Gilson 232-401 automated sampler (Gilson Medical Electronics Inc., Villiers le Bel, France). The HPLC equipment consisted of two Wallac 2258 pumps (Pharmacia Biotechnology, Uppsala, Sweden), a Beckman Gold C18-ultrasphere column (Beckman Instruments Inc., CA, USA), a Gilson C18 precolumn, and an ESA electrochemical detector (ESA Inc., MA, USA).

20.5.3 Arterial Preparations and Organ Bath Solutions

A detailed description of the technique adopted is provided elsewhere.[21] Briefly, the endothelium of the most distal ring was removed by gently rubbing the preparation with a jagged injection needle.[22] The rings were placed between stainless steel hooks (diameter 0.3 mm) and suspended in a 20-ml organ bath chamber in physiological salt solution, and aerated with 95% O_2 and 5% CO_2. The rings were initially equilibrated for 60 min at +37°C with a resting tension of 1.5 g. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph. The presence of intact endothelium in vascular preparations was confirmed by clear relaxation responses to 1 μM acetylcholine (ACh) in rings precontracted with 1 μM noradrenalin (NA), and the absence of endothelium by the lack of this response. If any relaxation was observed in endothelium-denuded rings, the endothelium was rubbed again.

20.5.4 Arterial Contractile and Relaxation Responses

After the equilibration period, the cumulative concentration-response curves for NA and potassium chloride (KCl) were determined. The next concentration of the agonist was added only when the previous level of response was stable. After the maximal response had been reached, the rings were rinsed with physiological salt solution (PSS) and allowed a 20-min recovery period at resting

tension. Endothelium-dependent arterial relaxation was tested after precontraction by NA and KCl. Rings were precontracted with either 1 μM NA or 50 mM KCl, and after full contraction, increasing concentrations of ACh were cumulatively added to the organ bath. Responses to ACh were then elicited in the presence of 3 μM diclofenac, and in the presence of diclofenac plus 0.1 mM N^G-nitro-L-arginine methyl ester (L-NAME). After removal of the vascular endothelium, the relaxation responses to sodium nitroprusside and isoprenaline were examined. The rings were precontracted with 1 μM NA, and upon full contraction, increasing concentrations of the relaxing agents were cumulatively added to the organ bath. After the maximal response had been reached, rings were rinsed with PSS and allowed a 20-min recovery period at resting tension. Thereafter, Ca^{2+} was omitted from the PSS, and the rings were contracted with 10 μM NA to empty the cellular Ca^{2+} stores.[23] When the maximal response had developed, the rings were rinsed with Ca^{2+}-free PSS, and once the resting tension was restored they were challenged with 125 mM KCl. When the response had reached a plateau, Ca^{2+} was cumulatively added to the organ bath. The procedure was then repeated in the presence of 0.5 nM nifedipine. A 30-min incubation was allowed after nifedipine was introduced.

20.6 RESULTS

20.6.1 Plasma Coenzyme Q Concentrations

After 8 weeks' supplementation with coenzyme Q_{10} (10 mg/kg/day), its plasma concentration was significantly higher in the supplemented than in the control group (290 nmol/l vs. 48 nmol/l, respectively, $P < 0.0001$). Coenzyme Q_9 concentrations in plasma were similar in both groups.

20.6.2 Function of Mesenteric Artery

In endothelium-denuded mesenteric artery rings, the relaxations elicited by sodium nitroprusside and isoprenaline were attenuated with aging when compared to young control rats (Figure 20.1, $P < 0.0001$). Aging was also associated with impairment of endothelium-mediated vasodilatation to ACh in rings precontracted with noradrenalin (Figure 20.2). When hyperpolarization of arterial smooth muscle was eliminated by precontraction induced by 50 mM KCl, no differences were found between the young and senescent groups in relaxation to ACh (data not shown). In endothelium-intact rings, the contractile responses to NA and to depolarization induced by KCl were less sensitive in old rats. Furthermore, young rats were more sensitive to contraction induced by cumulative Ca^{2+} during depolarization and more resistant to the inhibitory effect of nifedipine on this response (data not shown).

Supplementation with coenzyme Q_{10} clearly improved relaxation in response to isoprenaline ($P = 0.0001$) but did not affect the response to sodium nitroprusside (Figure 20.1). Supplementation also improved ACh-induced endothelium-mediated vasodilatation in NA-precontracted rings ($P = 0.01$). Cyclooxygenase inhibition with diclofenac enhanced the relaxation only in young rats, but abolished the difference between the coenzyme Q_{10}-supplemented and control groups (Figure 20.2). In KCl-precontracted rings there were no differences in relaxation to ACh between senescent rat groups. Likewise in contractile responses to NA, KCl, or Ca^{2+}, the senescent groups showed no differences (data not shown).

20.7 DISCUSSION

In our study, arterial function was studied *in vitro* in a selected segment of the superior mesenteric artery. The fact that blood vessels from different parts of the vasculature may differ substantially must be kept in mind when relating results to the whole vascular system. The main superior mesenteric

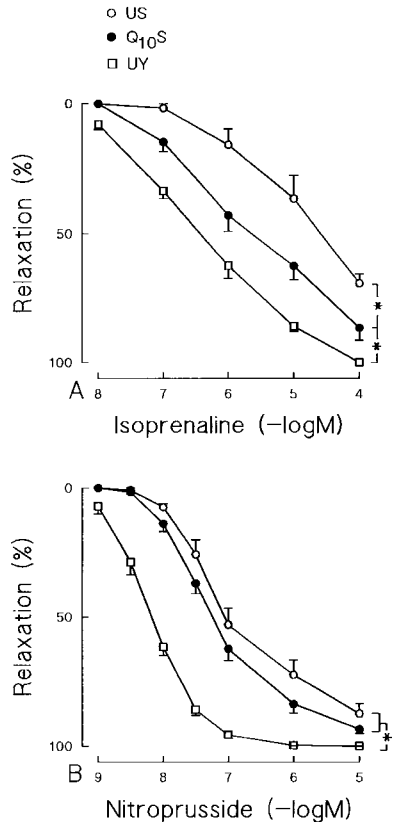

FIGURE 20.1 Relaxations to isoprenaline (A) and nitroprusside (B) after precontraction with noradrenaline (1 μM). The responses were elicited in isolated endothelium-denuded mesenteric arterial rings from unsupplemented senescent (US), Q_{10}-supplemented senescent ($Q_{10}S$) and unsupplemented young (UY) Wistar rats. Symbols indicate means with s.e.means, $n = 9$ in each group; $^*P < 0.05$, ANOVA for repeated measurements.

artery was used as a model in the experiment in view of its superior suitability for the present experimental setting, which required stable precontractions and reproducible relaxation responses.

The deterioration in arterial function with aging has been widely recognized.[9,10,14,17,18] In our study, there was a significant deterioration in endothelium-mediated vasodilatation as well as in β-adrenoceptor-mediated relaxation with aging. Also the vasodilation to the NO-donor nitroprusside was attenuated in senescent rats and the contractile responses were decreased compared with those in young rats. Endothelium-mediated and β-adrenoceptor-mediated vasodilations were improved, but not normalized to the same level as in young rats, by coenzyme Q_{10} pretreatment. Interestingly, inhibition of cyclooxygenase, which results in a decrease in the production of prostaglandins from the endothelium, abolished the difference in relaxation to ACh between the supplemented and the control group. Since PGI_2 is the major prostanoid produced in the vascular bed,[7] the enhancement of endothelium-dependent vasodilation after Q_{10} treatment was largely mediated by PGI_2. It is known that the stimulation of smooth muscle cell β-receptor leads to activation of adenylate cyclase and a subsequent increase in intracellular cAMP.[24] Furthermore, the cellular action of PGI_2 is exerted via binding to a cellular receptor that activates adenylate cyclase.[7] Thus, the observed improvement in endothelium-mediated vasodilation by Q_{10} treatment is possibly attributable to an increased endothelial production of PGI_2 or increased arterial smooth muscle sensitivity to agonists that induce vasorelaxation via an increase in cellular cAMP.

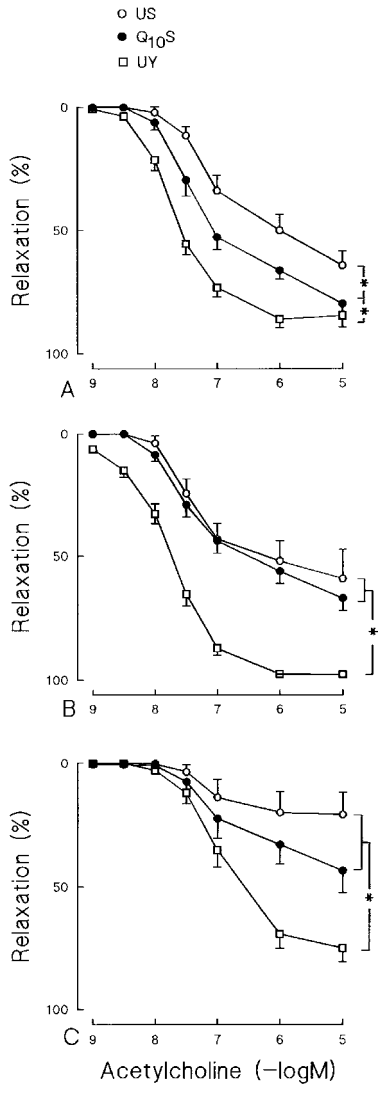

FIGURE 20.2 Relaxations to acetylcholine in noradrenaline (1 μM) precontracted isolated endothelium-intact mesenteric arterial rings from unsupplemented senescent (US), Q_{10}-supplemented senescent ($Q_{10}S$) and unsupplemented young (UY) Wistar rats. The relaxations were induced in the absence (A) and presence (B) of 3 μM diclofenac, in the presence of diclofenac and 0.1 mM N^G-nitro-L-arginine methyl ester (L-NAME; C). Symbols indicate means with s.e. means, $n = 9$ in each group; $^*P < 0.05$, ANOVA for repeated measurements.

Finally, it must be kept in mind that the method utilized in our study gives indirect evidence of the function of the mesenteric artery. The aim of future studies should be in confirming these results in other parts of the vasculature and furthermore to obtain direct evidence of the protection of arterial function by coenzyme Q.

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21 Implication of Coenzyme Q Depletion in Heart Transplantation

Anna Gvozdjáková and Jarmila Kucharská

CONTENTS

21.1 INTRODUCTION

Cardiac transplantation is an accepted therapy for patients with end-stage heart failure. Years of patient survival after heart transplantation depends on various factors, such as number of rejections, immunosuppression, production of free radicals, function of antioxidant defense systems, and mitochondrial bioenergetic stage.

Over the last years an enormous amount of succesful transplantations of vitally important organs in human medicine have been due to the discovery of a new immunosuppressive drug — cyclosporin A (Devaraj et al.,[4]). This drug is on one hand indispensable from the point of view of immunosuppressive therapy, yet on the other hand, it has side effects. Cyclosporin A is hepatotoxic; it damages the metabolism of bile acids and the production of bile, i.e., processes dependent on adenosine triphosphate. Even in very low concentrations, cyclosporin A damages mitochondrial functions. It inhibits mitochondrial permeability transition (MPT), which is characterized by progressive permeabilization of the inner mitochondrial membrane — from protons, ions, to small proteins, stimulated by osmotic support (Kowaltovski and Vercesi[14]).

Patients living with transplanted hearts require continual complex specialized medical care and complex therapy for a lifetime. After heart transplantation, patients have to be regularly checked for possible transplant rejection, monitored by immunosuppressive therapy, and need effective prevention and therapy for infectious diseases (Fabián et al.[7]).

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In spite of effective immunosuppression, acute rejection of the transplanted organ is one of the greatest problems in the first year after heart transplantation. The exact pathobiochemical mechanisms that participate in rejection development are not fully known. Although histological evaluation of endomyocardial biopsy (EMB) is one of the standard diagnostic methods for detection of the degree and dynamics of rejection of the transplanted heart, new methods of diagnosing early rejection symptoms are being developed. EMB is demanding, uncomfortable for the patient, and can be a cause of complications.

The aim of our studies was to contribute to the development of new diagnostic methods for early and quick detection of human transplanted heart rejection, focusing on the endogenous level of coenzyme Q_{10} in EMB and on possible changes of EMB mitochondrial bioenergetics.

21.2 FACTORS OF ACUTE REJECTION OF THE TRANSPLANTED HEART

Infections represent major complications for transplant patients. Inflammatory processes are involved in mechanisms of acute rejection of the transplanted heart. Inflammatory cells like B and T lymphocytes and macrophages were found in transplanted hearts early after transplantation, even without any acute clinical rejection symptoms (Hruban et al.,[11] Rose et al.,[24]). The authors focused on immunocytochemical markers of activation in cardiac transplant rejection. Neutrophils activated by inflammatory processes were found to participate in uncontrolled free radical production (Karlsson et al.,[13]).

The importance of stress proteins or heat shock proteins (Hsp) has been established in various conditions such as inflammation, infection, autoimmune disease, and tumor immunity. The role of stress proteins and their correlation with the degree of cellular rejection of the transplanted human heart was first documented by Moliterno et al.[21] During rejection, increased Hsp expression is a part of the stress response. Hsp expression increases in three stress stages: The first is a physiological stress secondary to the trauma of the transplant procedure and ischemia/reperfusion injury (Hsp60, Hsp72). The second is associated with infiltration of lymphocytes in the allograft. Finally, during the third stage, the stress response to the inflammatory processes associated with rejection leads to increased expression of Hsp, including lower molecular weight proteins, which may represent proteolytic degradation of Hsp (Duquesnoy[5]).

Most transplant patients have elevated pulmonary artery pressure, with right ventricular hypertrophy and dilatation. The latter could be the cause of increased collagen I. Increased intracellular matrix proteins — collagen I and fibronectin — in the human transplanted heart during the first 70 days after heart transplantation were first shown by Schacherer et al.[25] Circulation antibodies to the extracellular matrix were found in transplant recipients even if no acute rejection was observed (Rose et al.[24]).

21.3 PATHOBIOCHEMICAL MECHANISMS OF REJECTION OF THE TRANSPLANTED HEART

The following pathobiochemical mechanisms could be involved in acute rejection development of the transplanted heart: increased free oxygen radical production, diminished antioxidant defense activities, and damaged heart mitochondrial function.

21.3.1 Free Radicals — Antioxidants

There is a dynamic balance between reactive oxygen species (ROS) production and antioxidant defense systems. Uncontrolled ROS production induces structural and functional alterations of cellular membranes, damage of polyunsaturated fatty acids, proteins, and deoxyribonucleic acid. Each cell contains antioxidant systems that protect tissues from oxidative insults, cell necrosis, and apoptosis. Coenzyme Q_{10}, alpha-tocopherol and beta-carotene belong to the nonenzymatic lipid-soluble antioxidants. Nutritional elements (Mg, Se, Zn, Cu) participate in the defense antioxidant

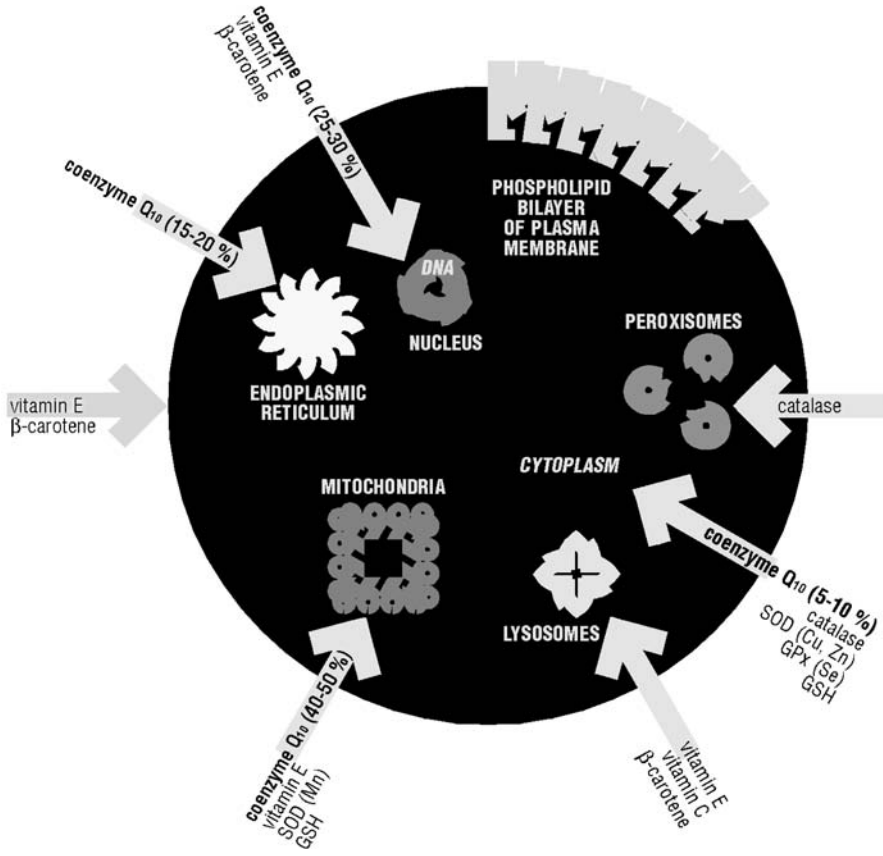

FIGURE 21.1 Antioxidants in the cell (Gvozdjáková, A., Gvozdják, P., Ateliér 2, 1997).

systems of the human body. Enzymatic antioxidant defense can be provided by superoxide dismutase, catalase, and glutathione peroxidase (Figure 21.1).

21.3.1.1 Peroxidation of Lipids

In patients after heart transplantation (HTx-pts), plasma malondialdehyde (MDA) is significantly increased in comparison with the healthy group. There is no correlation between the levels of malondialdehyde and degree of transplant rejection (Gvozdjáková et al.) (Figure 21.2).[10]

21.3.1.2 TBARS

Substances that react with thiobarbituric acid (TBARS) are products of lipoperoxidation (LPO). TBARS in plasma in HTx-pts (23 patients) were assessed four times per year and statistically evaluated in comparison with a control group of 50 healthy persons. The four time periods were classified as follows: 1 = October–December, 2 = January–March, 3 = April–June, 4 = July–September (Table 21.1). Significantly increased concentrations of TBARS in plasma of HTx-pts in comparison with healthy subjects ($P < 0.001$) in the period of October–March may be associated with lower intake of vitamins with antioxidant properties (Pecháň et al.[23]).

21.3.1.3 Total Antioxidant Status

In HTx-pts, total antioxidant status (TAS) is significantly decreased in comparison with healthy subjects during October–March. This parameter was not measured in the period April–September

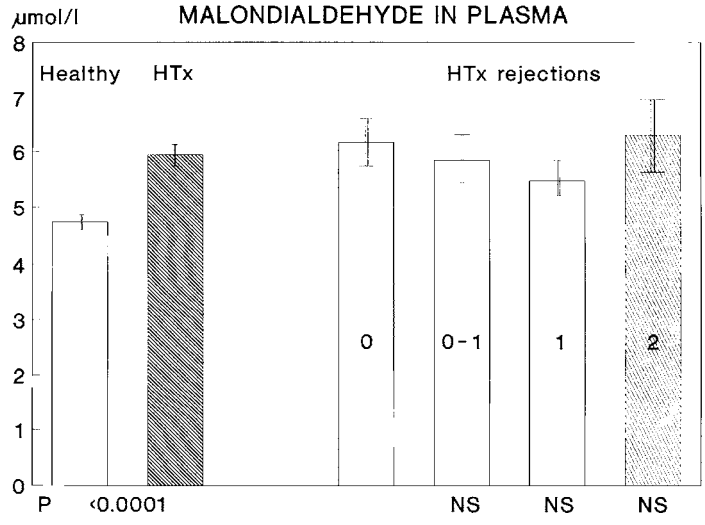

FIGURE 21.2 Plasma malondialdehyde in HTx-pts.

TABLE 21.1
TBARS in Plasma of HTx-pts

	Healthy	Heart Transplnted Patients				
		1–4	1	2	3	4
mean	2.020	2.442	3.610	3.050	1.670	1.440
± s.e.m.	0.509	0.628	1.012	0.562	0.523	0.416
P			<0.001	<0.001	<0.05	<0.01

Explanation:

TBARS = ng/l
s.e.m. = standard error of mean
P = statistical significance
1–4 = periods of year
1 = October–December
2 = January–March
3 = April–June
4 = July–September

(Table 21.2). Decreased TAS and increased ROS production can contribute to the development of rejection of the transplanted heart. TAS was determined using the kit from Randox (UK).

21.3.2 Coenzyme Q_{10}

Coenzyme Q_{10} (CoQ_{10}) is produced in every cell and is a key part of mitochondrial respiratory chain connected with oxidative phosphorylation. CoQ_{10} is essential for ATP synthesis. It is also an important natural antioxidant (Beyer,[1] Lenaz et al.[17]) associated with human low density lipoproteins (LDL). In its reduced form, it protects lipoproteins from peroxidative damage more effectively than alpha-tocopherol (Stocker et al.,[29] Ernster and Forsmark[8]). A model for the role of ubiquinones in biological membranes has been proposed by Crane.[3] Decreased levels of CoQ_{10} were confirmed in some types of cardiomyopathies and in the failing heart (Mortensen et al.,[22] Folkers[8]). Only sporadic

TABLE. 21.2
TAS in Plasma of HTx-pts

	Healthy	Heart Transplanted Patients				
		1–4	1	2	3	4
mean	1.856	1.222	1.380	1.064	–	–
± s.e.m.	0.303	0.170	0.244	0.096	–	–
P			<0.001	<0.001		

Explanation:

TAS = total antioxidant status (mmol/l)
s.e.m. = standard error of mean
P = statistical significance
1–4 = periods of year
1 = October–December
2 = January–March
3 = April–June
4 = July–September

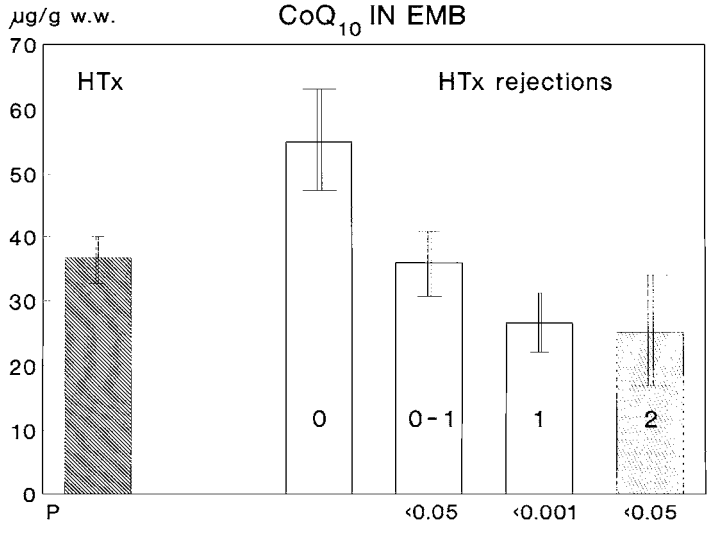

FIGURE 21.3 Relationship between CoQ_{10} content in EMB and degree of rejection of human transplanted heart.

and controversial data have been published with respect to the endogenous level of CoQ_{10} in relation to rejection of the transplanted heart. In the study of Karlsson et al.,[13] blood and myocardial levels of CoQ_{10} diminished in HTx-pts. No changes were found during rejection development in heart transplanted patients (Sehested et al.[28]). These authors observed no significant differences between plasma levels in 68 patients awaiting heart transplantation and in 42 posttransplanted patients without rejection. No correlation was found between plasma levels and biopsy concentration of CoQ_{10} in the transplanted heart.

We found correlation between endogenous myocardial CoQ_{10} level and the degree of rejection of the human transplanted heart (Kucharská et al.,[15] Kucharská et al.,[16] Gvozdjáková et al.[10]) (Figure 21.3). Mean age of patients was 45 years, range 16 to 63 years. Twenty-eight EMB were divided according to the histologically confirmed degree of rejection; 0 = without rejection, 0-1 = incipient rejection,

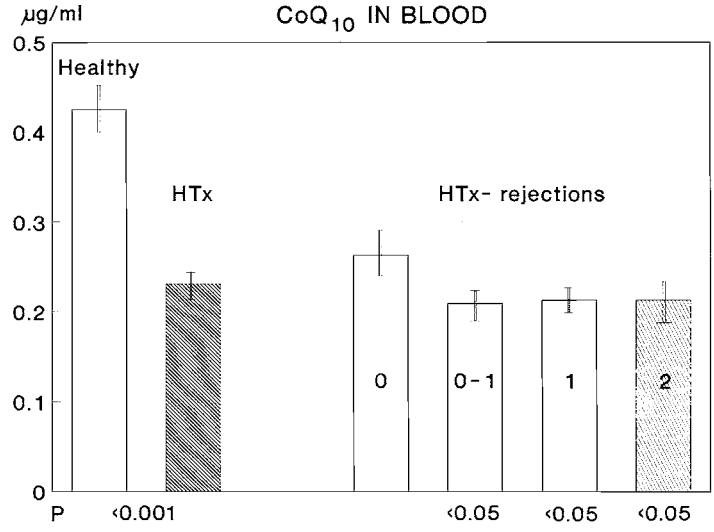

FIGURE 21.4 Relationship between CoQ_{10} in whole blood and degree of rejection in HTx-pts.

1 = mild rejection, 2 = moderate rejection. The mean CoQ_{10} concentration in EMB in HTx-pts was 36.7 ± 3.72 ug/g w.w. The results were statistically evaluated according to the degree of rejection and compared with the group of patients without rejection. In the incipient rejection group, the concentration of CoQ_{10} was significantly decreased (35.9 ± 5.19 ug/g w.w. vs. 54.9 ± 7.79 ug/g w.w.-rejection 0, $P < 0.05$). In rejection 1, CoQ_{10} in EMB was 26.6 ± 4.65 ug/g w.w. ($P < 0.01$). In rejection 2, content of CoQ_{10} in EMB was 25.2 ± 8.74 ug/g w.w. ($P < 0.05$).

The relationship between CoQ_{10} whole blood level and degree of rejection in HTx-pts is shown in Figure 21.4. The mean value of CoQ_{10} determined in whole blood of 50 healthy subjects was 0.425 ± 0.026 ug/ml. This value is at the lower limit of the reference range in healthy persons reported in developed countries (0.4 to 1.0 ug/ml). This can be due to a lower intake of CoQ_{10} in the diet of the Slovak population as well as to factors influencing biosynthesis and utilization of CoQ_{10}. Blood concentration of CoQ_{10} in HTx-pts without rejection was evaluated in comparison with rejection groups of patients. In patients with degree 0 to 1, blood CoQ_{10} concentration was 0.209 ± 0.01 ug/ml ($P < 0.05$). In degree 1, it was 0.213 ± 0.014 ug/ml ($P < 0.05$), and in degree 2, blood CoQ_{10} level was 0.213 ± 0.023 ug/ml ($P < 0.05$).

The relationship between incipient rejection, developed rejection, and endogenous level of CoQ_{10} in EMB of HTx-pts provokes the following questions:

a. What is the cause for these relationships?

b. Which other pathobiochemical mechanisms are directly dependent on CoQ_{10} level in the transplanted heart?

We attempted to address these questions by using methodical approaches for early and rapid determination of rejection development, focusing on mitochondrial respiratory chain function and energy production in EMB of HTx-pts.

21.3.3 Mitochondrial Medicine

Mitochondria are essential eukaryotic organelles performing many important physiological cellular functions. They are special organelles for respiration and oxidative phosphorylation, producing almost 90% of energy in the cell. This energy is necessary for the continual function of the heart muscle. In addition to these functions, mitochondria synthetize heme, lipids, amino acids, and nucleotides and they maintain homeostasis of inorganic ions. It has been demonstrated that with destruction of cells, mitochondria are also destroyed. Mitochondria contain 5 to 10% of cellular

proteins. Import of proteins from cytoplasm into mitochondria is the main mechanism of mitochondrial biogenesis, as has been shown only recently (Schatz[26]).

While most of oxygen consumed by mitochondria is reduced to water at complex IV, about 1 to 2% of oxygen aquires an electron directly, generating superoxide ions, which are converted to H_2O_2 and $^{\cdot}OH$ radical. These chemical oxidants are normal products of oxidative processes. They may be harmful when produced in increased amounts and are not neutralized by the naturally occurring antioxidants. Karbowski et al.[12] reported that excessive amounts of free radicals provoke megamitochondria formation. Mitochondria become enlarged to various degrees with consequent lowering of the rate of oxygen consumption and phosphorylating abilities. Mitochondrial function is differently affected by oxidative stress (Cardoso et al.[2]). Leakage of reactive oxygen species may lead to damage of the mitochondrial membrane, proteins, and DNA and to inhibition of oxidative phosphorylation (Luft,[18,19] Luft, and Landau[20]). Radicals also arise from destruction of cells during chronic infections, inflammations, and the effect of adriamycin A. It has not been fully established whether increased ROS production and a deficit of CoQ_{10} affect the development of rejection of the transplanted heart.

For many years it was not possible to perform metabolic studies of respiratory chain and oxidative phosphorylation in mitochondria from the human heart muscle. Studies were carried out on various models of heart muscle mitochondria damage in experimental animals. Significant changes have occurred in the direction of this research over the past decade. Veksler et al.[30] applied a new methodological approach in mitochondrial studies, using only 2 to 10 mg of tissue. We used the method of skinned fibers for study of bioenergetic and respiratory properties of mitochondria in human endomyocardial biopsies of patients awaiting heart transplantation as well as after heart transplantation (Gvozdjáková et al.[9]).

The purpose of these studies was to contribute to the explanation of the relationship between incipient and developed rejection of the transplanted heart and endogenous levels of CoQ_{10}, respiration, bioenergetics, and the creatine kinase system of mitochondria in EMB. Bioenergetic mitochondrial studies and studies concerning CoQ_{10} concentrations in the human heart are among to unique investigations published in the world literature. No studies have come to our attention before we published relevant information on the involvement of mitochondrial respiratory chain in rejection of transplanted heart.

More information accumulated on skeletal muscle mitochondria in patients with various types of mitochondrial diseases, such as mitochondrial myopathies and mitochondrial encephalomyopathies. In 1994, the Nobel Symposium 90 was focused on mitochondrial diseases, including mitochondrial cardiomyopathy. Of different biochemical changes in mitochondria (more than 120 recognized types, Scholte[27]) most attention has been focused on the function of mitochondrial respiratory chain, energy production, endogenous concentration of CoQ_{10}, and oxidative stress.

21.3.3.1 Respiratory Chain — Oxidative Phosphorylation

The main oxidative metabolism in the cell, yielding ATP from ADP and Pi, is electron transport of the respiratory chain located in the inner membrane of mitochondria. The respiratory chain is organized as four protein complexes (I, II, III, IV) that form part of the structure of the inner mitochondrial membrane. These complexes contain mobile components — ubiquinone and cytochrome *c*. The transport of electrons from NADH and $FADH_2$, production of electrochemial potential, and proton gradients between two sides of the inner mitochondrial membrane are necessary for the synthesis of ATP via mitochondrial ATPase (complex V). Electrons are carried from complex I and II to complex III by coenzyme Q, and from complex III to complex IV (cytochrome oxidase) by cytochrome *c*. Mitochondrial ATP is exchanged for cytosolic ADP.

For the studies of mitochondrial function in EMB in HTx-pts we used 2 to 5 mg of tissue. Skinned fibers were prepared by permeabilization of the tissue with saponine (Veksler et al.[30]) and measurements of mitochondrial respiration, ATP production, and creatine kinase system activity were performed and CoQ_{10} and alpha-tocopherol content determined. Mitochondrial respiration

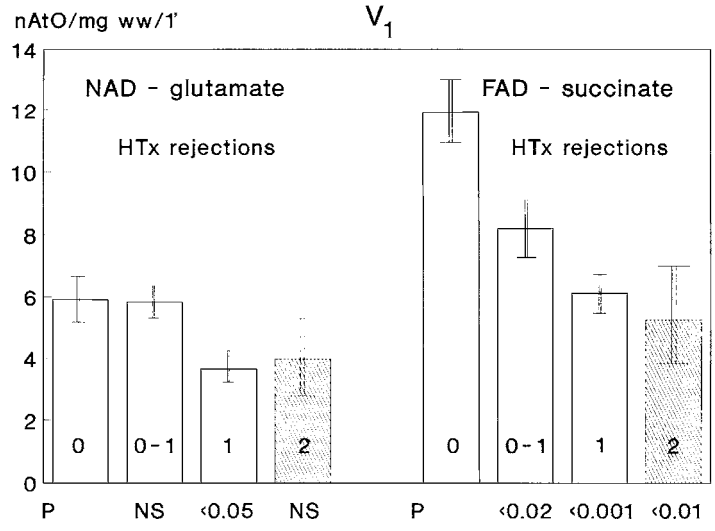

FIGURE 21.5 Basal mitochondrial respiration in EMB of HTx-pts in relation to degree of rejection.

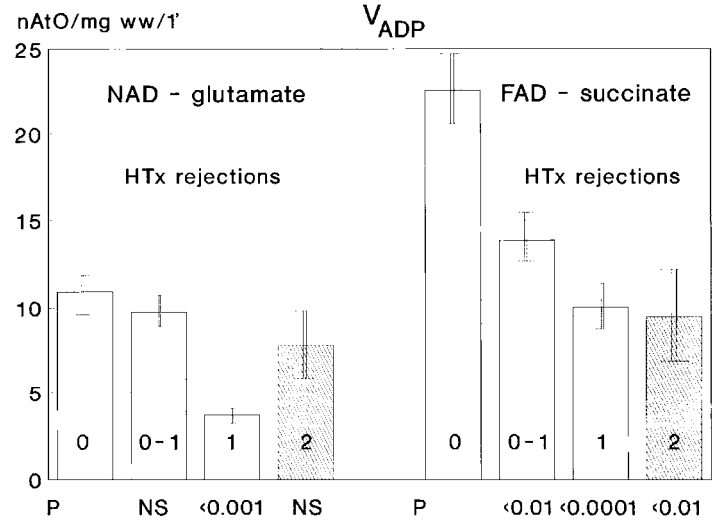

FIGURE 21.6 Stimulated mitochondrial respiration in EMB of HTx-pts in relation to degree of rejection.

and creatine kinase activity was measured by Oxygen Gilson 5/6H (USA) using Clark oxygen electrode; CoQ_{10} and alpha-tocopherol content were determined by HPLC method (LKB, Pharmacia). The number of EMB for mitochondrial study was 60, the number of patients was 34. The number of EMB from one patient was 1 to 4.

Basal (V_1) and ADP stimulated (V_{ADP}) mitochondrial respiration in EMB of HTx-pts in relation to degree of rejection, using NAD- and FAD-linked substrates is demonstrated in Figures 21.5 and 21.6. Using NAD-substrate (glutamate) we showed that mitochondrial respiration and ATP production were damaged in degree 1 rejection. The FAD-site of respiratory chain was more sensitive to rejection development, with respiration inhibited already in degree 0 to 1 of rejection. In EMB of HTx-pts, we did not find any relationship between specific activity of cytochromeoxidase and degrees of rejection of transplanted heart (Figure 21.7).

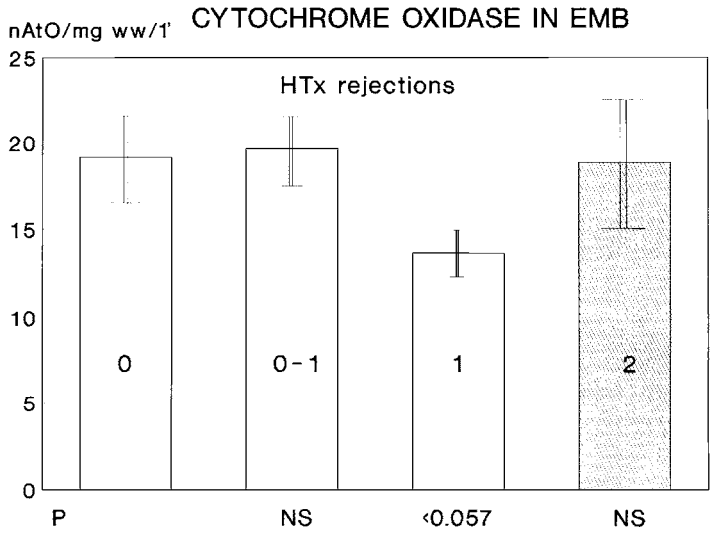

FIGURE 21.7 Cytochrome oxidase in EMB of HTx-pts in relation to degree of rejection.

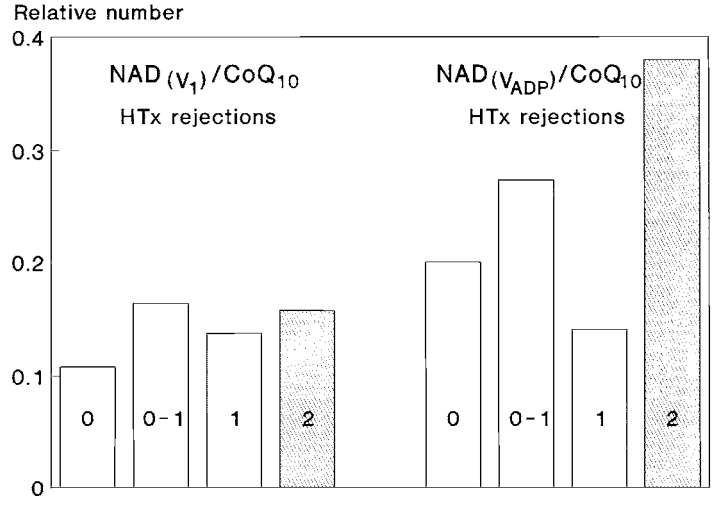

FIGURE 21.8 Relationship between NAD (V_1, V_{ADP})/CoQ_{10} ratio and degree of rejection of transplanted heart.

At this time it is unclear whether diminished CoQ_{10} concentration in EMB with degrees of rejection in HTx-pts plays a direct role in the electron transport for complexes I and II of respiratory chain or whether CoQ_{10} serves for pathobiochemical processes. Figrue 21.8 demonstrates that NAD (V_1, V_{ADP})/CoQ_{10} ratios were not dependent on the content of CoQ_{10} in the tissue.

Using FAD-substrate (succinate) we found a different situation. The FAD (V_1,V_{ADP})/CoQ_{10} ratio is constant. This means that CoQ_{10} limits the function of complex II of the respiratory chain at the site of FAD-Coenzyme Q (Figure 21.9). The changed NAD/CoQ_{10} ratio suggests that the defect of complex I of the respiratory chain at the site of NAD-Coenzyme Q may have occurred upstream of the respiratory chain, i.e., in the Krebs cycle or CoQ_{10} serves for glycolytic NADH oxidation.

The question remains as to whether the activity of mitochondrial cytochrome oxidase depends on CoQ_{10} content. We found that the function of the mitochondrial respiratory chain at the sites of complexes I and II of the respiratory chain does not depend on the activity of mitochondrial

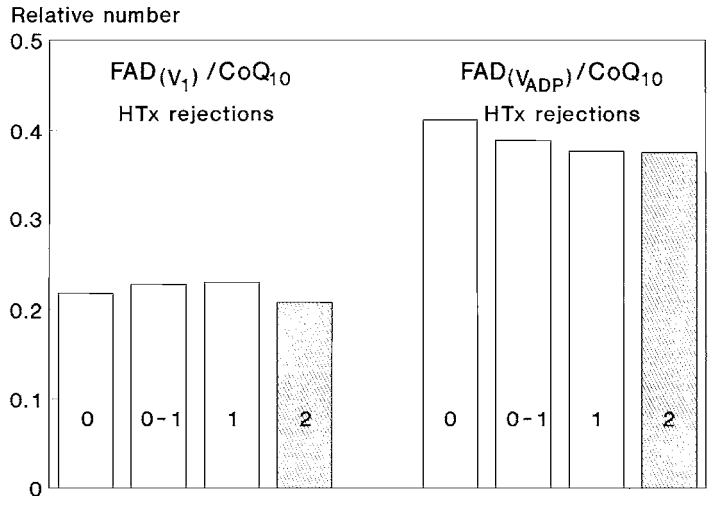

FIGURE 21.9 Relationship between FAD (V_1, V_{ADP})/CoQ_{10} ratio and degree of rejection of transplanted heart.

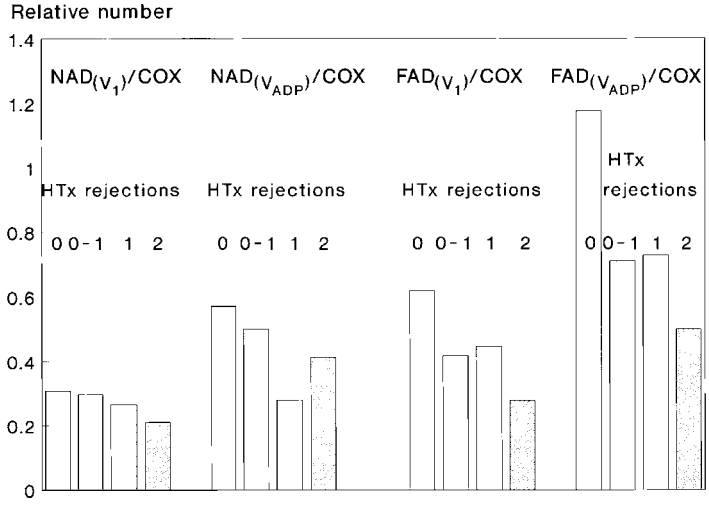

FIGURE 21.10 Relationship between complexes I and II and complex IV of the respiratory chain and degree of rejection of the transplanted heart.

cytochrome oxidase of the transplanted heart. There is no correlation between the development of rejection and the NAD- or FAD/cytochrome oxidase ratio (Figure 21.10).

21.4 CONCLUSIONS

1. Our results strongly indicate that the pathobiochemical mechanisms of rejection of human transplanted heart also involve bioenergetic processes of heart muscle mitochondria.
2. Determination of CoQ_{10} levels in EMB from HTx-pts appears to be a new methodological approach to study the onset and development of rejection of the transplanted heart.
3. In patients after heart transplantation, diminished CoQ_{10} levels are directly associated with mitochondrial respiration and bioenergetics of the heart muscle:

a. The function of the respiratory chain at the site of complex I (NAD-linked respiration) does not depend on the level of CoQ_{10}. We suppose that the damage has already occurred at the stage of glycolysis or at the level of the Krebs cycle.
b. The function of the respiratory chain at the site of complex II (FAD-linked respiration) is limited by the level of CoQ_{10}.
c. The activity of mitochondrial cytochrome oxidase is independent of the CoQ_{10} level.

4. Treatment of patients after HTx with CoQ_{10} could improve heart mitochondrial function, support the antioxidant defense system, and thus participate in prevention of rejection development. We have hope for this perspective for the new millennium.

ACKNOWLEDGMENTS

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Section 3C

Liver Injury

22 A Role for Coenzyme Q in Alcoholic Liver Disease?

Simon Eaton, Christopher O. Record, and Kim Bartlett

CONTENTS

22.1 INTRODUCTION

In spite of the large volume of literature on the involvement of coenzyme Q (CoQ) in human pathology, there is surprisingly little work reported on CoQ in alcoholic liver disease. In this review, we will consider the biochemical basis of alcoholic liver disease, the evidence for the involvement of free-radical processes and nutritional factors in alcoholic liver disease, and the evidence, both direct and indirect, for CoQ involvement in alcoholic liver disease.

Chronic alcohol consumption is hepatotoxic in man and can lead to an accumulation of hepatic triglycerides. This steatosis is mainly macrovesicular and perivenous in distribution. Alcohol-induced fatty liver has been considered to be a benign condition,[1] however there is increasing evidence from follow-up studies in humans that fatty liver may be important in progression to fibrosis and cirrhosis.[2,3] The view of fatty liver as a benign condition stems, in part, from the observation that rats fed alcohol, by means of one of the most extensively applied protocols (the Lieber-DeCarli diet), do not progress to fibrosis and cirrhosis.[4] In addition, the response of individual humans to alcohol is highly variable and by no means do all chronic alcohol abusers have steatosis, fibrosis, or cirrhosis. It has been suggested that both genetic susceptibility[5] and nutritional factors (see below) may have a role in the heterogenous long-term effects of alcohol in man, and that therefore animal models are of limited relevance to investigations of the pathogenesis of human alcoholic fatty liver. Accordingly, in the present review we limit the literature cited, where possible, to human studies. In spite of the plethora of studies on alcohol-induced fatty liver, the pathogenesis is not well understood;[6] neither are the factors that may cause progression of disease to fibrosis/cirrhosis.

22.2 ALCOHOLIC LIVER DISEASE AS A FREE-RADICAL DISEASE

Ethanol metabolism in hepatocytes *in vitro* results in the generation of reactive oxygen species,[7,8] and in animal models of alcoholic liver disease, the activity of alcohol-induced cytochrome P4502E1, which can generate superoxide, hydroxyl, and hydroxyethyl radicals, appears to be an

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important determinant of both liver damage and hydroxyethyl radical production.[9] Initial studies in humans failed to detect an increase in thiobarbituric acid reactive substances or malondialdehyde resulting from *in vivo* lipid peroxidation, or electron paramagnetic resonance-determined free radicals in alcoholics.[10] However, more recent studies using alternative indices of lipid peroxidation, such as ethane elimination in breath, have provided evidence for increased lipid peroxidation in alcoholics.[11,12,13] Furthermore, lipid peroxidation has been hypothesized to play a role in the pathogenesis of alcoholic liver fibrosis[14] and steatohepatitis.[15] As iron exacerbates free-radical production, the combination of ethanol and iron has been proposed as critical in the progression of steatosis to fibrosis and cirrhosis[16] and iron status is important in alcoholic liver disease in general.[17] Studies in primates also support the relationship between lipid peroxidation and alcohol-induced liver damage.[18,19]

22.3 IMPORTANCE OF NUTRITION IN ALCOHOLIC LIVER DISEASE

Nutrition is of great importance in alcoholic liver disease, to the extent that some authors regard ethanol itself as nonhepatotoxic, and that the liver disease associated with chronic ethanol intake is caused purely by inadequate nutrition, in both animal models and human disease.[20,21] Most authors now, however, regard ethanol as hepatotoxic and that the degree of hepatotoxicity is modified by nutritional factors.[4,22] Not surprisingly for a disease associated with free-radical production, dietary antioxidant intake is of great importance in alcoholic liver disease. Particular interest has been shown in a possible relationship between vitamin E status and alcoholic liver disease. However α-tocopherol status of alcoholics has been variably reported as increased, decreased, or unchanged compared to controls (e.g., [23–25], [26–28]). These differences may be due to the degree of liver damage in the studied groups and/or whether α-tocopherol levels are normalized to plasma lipid levels. Studies on the vitamin C, selenium, and β-carotene status of alcoholics have been also reported.[29,30] In addition, there have been very many studies indicating an amelioration of alcoholic liver damage by various antioxidants in experimental animals [31,32]

22.4 BIOCHEMICAL PATHOGENESIS OF ALCOHOL INDUCED FATTY LIVER

Hepatic metabolism of alcohol results in an increase in the cytosolic [$NADH/NAD^+$],[33] which could lead to (i) inhibition of β-oxidation and fat accumulation via mitochondrial [$NADH/NAD^+$],[34] or (ii) increased cytosolic ratio of [glycerol-3-phosphate]/[dihydroxyacetone phosphate] and enhanced esterification. [35,36] However, in animal studies, addition of oxidants such as methylene blue to the diet does not prevent alcoholic fatty liver[37] and hepatic redox changes are attenuated to levels seen in control animals after some months of alcohol feeding without a concomitant lowering of triglyceride levels.[38] Other possible causes of hepatic steatosis are direct effects on the esterification pathway,[39,40] cytosolic fatty acid binding protein concentration,[41] and decreased VLDL secretion.[42] The etiology of alcohol-related steatosis does not appear to be mediated by effects on lipolysis of hepatic triacylglycerols,[43] or by *de novo* lipogenesis.[44,45] Direct impairment of the mitochondrial β-oxidation of fatty acids by alcohol could cause triglyceride accumulation. Although impaired β-oxidation has been observed *in vivo* in man,[46–49] the results obtained from these studies require careful interpretation (see [6, 50]). Two studies on human tissues, which compared biopsies from alcoholic and normal subjects showed that there was no difference in β-oxidation flux between alcoholics and controls [50,51] but provided evidence that the respiratory chain and the citric acid cycle may have been impaired. Changes in the activity of the mitochondrial respiratory chain could be responsible for both diminished β-oxidation and citric acid cycle activities. Various studies have found diminished respiratory chain activity in alcoholic fatty liver [52–55] and this has been attributed to lowered transcription of mitochondrially encoded subunits of respiratory chain proteins.[56] However, a human study of the activity of respiratory chain complexes in alcoholic fatty liver found unchanged

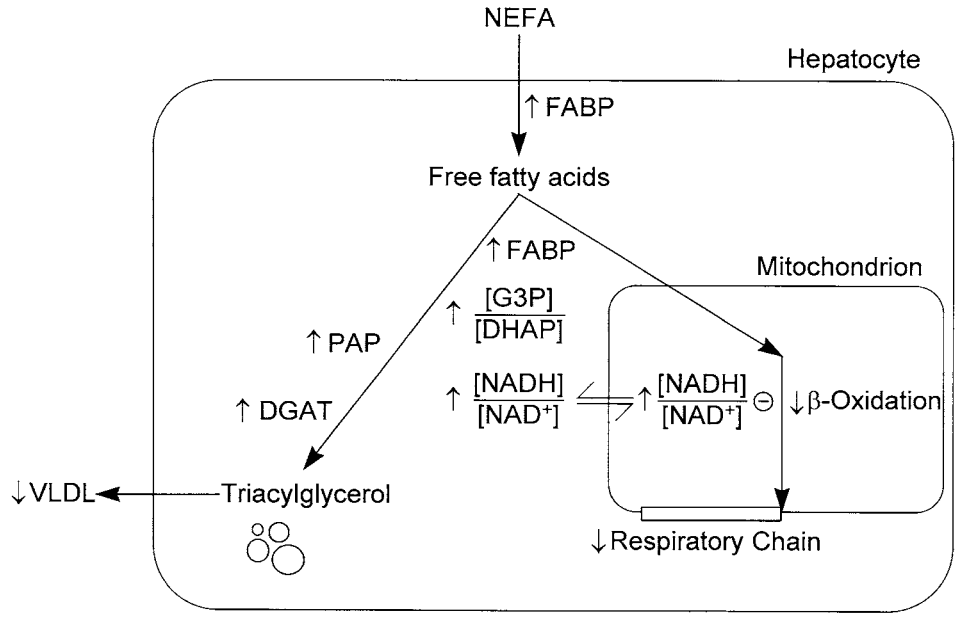

FIGURE 22.1 Biochemical effects of alcohol on fatty acid metabolism. Abbreviations: NEFA, nonesterified fatty acids; PAP, phosphatidate phosphohydrolase; DGAT, diacylglycerol acyltransferase; VLDL, very-low density lipoprotein; FABP, fatty acid binding protein; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate. From [6] with permission.

activity of complexes II and IV.[57] More recently, damage to mitochondrial DNA by alcohol has been detected in animals[58,59] and a higher occurrence of deletions within mitochondrial DNA in alcoholic patients has been observed.[60,61,62] These factors are indicated in Figure 22.1.

22.5 COENZYME Q IN ALCOHOLIC LIVER DISEASE

In order to examine the possible inhibition of mitochondrial β-oxidation in alcoholic fatty liver, we incubated hepatic mitochondria from alcoholics with [U-^{14}C] palmitic acid and measured the flux and intermediates of β-oxidation. Although there was no difference in β-oxidation flux between alcoholics and controls, there was a significant accumulation of 3-hydroxyacyl-CoA esters in mitochondria from patients with alcoholic fatty liver (Figure 22.2).[50] This could be caused by (i) decreased activity of the long-chain 3-hydroxyacyl-CoA dehydrogenase of mitochondrial β-oxidation, (ii) diminished amounts of intramitochondrial NAD, (iii) increased intramitochondrial NADH/NAD$^+$ due to ethanol, (iv) lowered activity of the complexes of the respiratory chain, or (v) lowered amounts of CoQ. However, 3-hydroxyacyl-CoA dehydrogenase activity was found to be similar to controls. Ethanol was not present during the incubations and lowering of intramitochondrial NAD$^+$ is unlikely, so decreased activity of the complexes or amount of CoQ appear to be the most likely explanations. Mitochondrial CoQ has not been measured in human alcoholic liver disease. Various animal studies in which mitochondrial CoQ was measured have given conflicting results: Bernstein and Penniall demonstrated a significant decrease in the CoQ content of mitochondria isolated from rats fed ethanol for 40 to 60 days[52] but there were no significant differences in mitochondrial CoQ content between alcohol-fed rats or pair-fed controls in the studies of either Koch et al.[63] or Thayer and Rubin.[64] Other studies have indicated that there is a depletion of CoQ in the Golgi apparatus of ethanol-fed rats;[65,66] as the Golgi apparatus is involved in synthesis and/or processing of endogenous CoQ,[67] this decrease may reflect an alcohol-induced decrease in hepatic CoQ biosynthesis.

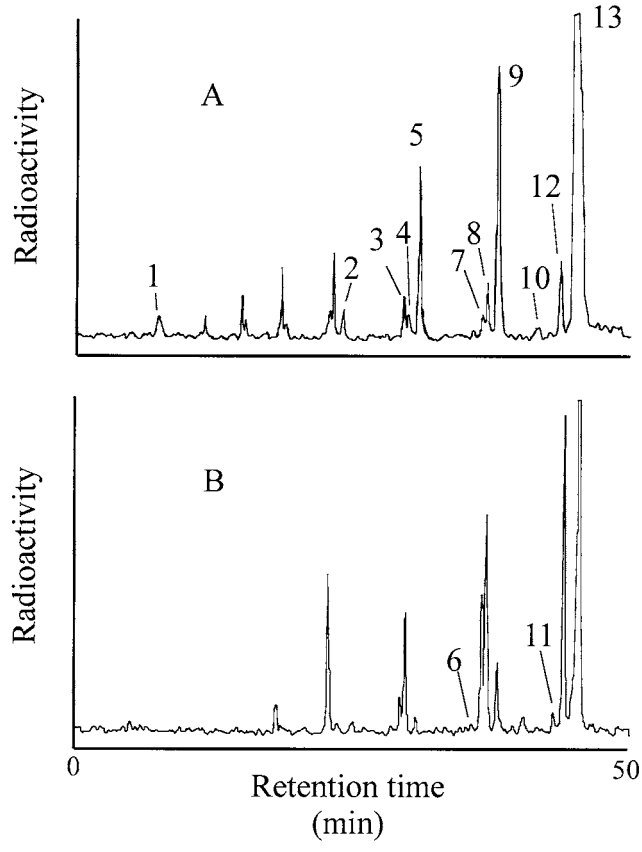

FIGURE 22.2 Sample radio-HPLC chromatograms showing the accumulation of CoA esters from [U-^{14}C] hexadecanoate by human liver mitochondria. [A] Normal subject [B] patient with alcoholic fatty liver. Peaks are labeled: (1) acetyl-, (2) 5-decanoyl-, (3) dodec-2-enoyl-, (4) 3-hydroxytetradecanoyl-, (5) dodecanoyl-, (6) tetradec-3-enoyl, (7) tetra-dec-2-enoyl-, (8) 3-hydroxyhexadecanoyl-, (9) tetradecanoyl-, (10) unknown, (11) hexadec-3- enoyl-, (12) hexadec-2-enoyl-, (13) hexadecanoyl-. Reproduced with permission from S. Eaton et al. 1996 *Clinical Science*, 90: pages 307–313. © Biochemical Society and Medical Research Society [50].

There have been very few studies measuring the concentration of CoQ in patients with liver diseases. To our knowledge, the only study comparing the plasma levels of CoQ in alcoholics and control patients is the study of Bianchi et al.[68] that demonstrated a dramatic decrease in plasma CoQ in cirrhotic patients and a smaller decrease in plasma CoQ in noncirrhotic alcoholics compared to controls (Figure 22.3). There was a significant negative correlation of plasma CoQ levels with total bilirubin but there was no correlation with other indices of liver function. However, careful interpretation of differences in plasma CoQ levels is necessary.[69] In another study, it was suggested that the redox state of plasma CoQ provides a marker of oxidative stress and that although the total plasma CoQ level was unchanged in hepatitis, cirrhosis, and hepatoma, the plasma CoQ pool was more oxidized in these patient groups, suggesting greater oxidative stress.[70] However, the redox state of plasma CoQ in alcoholics was not reported. Similarly, in liver tissue from alcohol-fed rats, there was a significant decrease in liver reduced CoQ, but similar levels of oxidized CoQ, compared to control rats.[71] This difference was exacerbated by iron overload. Another study reported significantly decreased liver CoQ in the reduced form compared to control animals but oxidized CoQ was not measured.[31]

As discussed above, the interaction of nutrients and/or antioxidants is important in experimental studies on the pathogenesis of alcoholic liver disease as well as in human pathology.

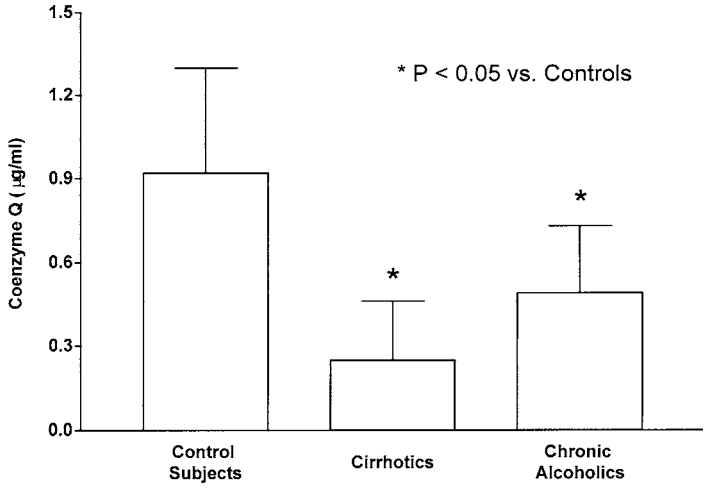

FIGURE 22.3 Coenzyme Q_{10} plasma levels in relation to liver disease and alcohol abuse. Data mean ± S.D., redrawn from [68] with permission.

A study by Loop et al.[72] showed that in rats fed ethanol at 20% of energy intake (lower than the Lieber-DeCarli diet, and designed to model chronic moderate alcohol intake), there was no alteration in hepatic CoQ levels but a significant decrease in hepatic vitamin E and retinol palmitate. When ethanol-fed rats were supplemented with CoQ at very high levels (approximately 37.5 mg CoQ_{10}/kg body weight/day), vitamin E, but not retinol palmitate, levels returned to control levels. When rats were fed ethanol at 35% of energy intake (close to the ethanol intake of the Lieber-DeCarli diet), there was a significant increase in liver CoQ compared to control rats, but not to pair-fed controls.[73] Other groups have shown a possible protective effect of CoQ against ethanol-induced hepatic triglyceride increases,[74] acetaldehyde levels in blood and liver of alcohol fed animals,[75] and increases in urinary products of lipid peroxidation in ethanol-fed animals.[76] These studies are probably indicative of a general antioxidant action of CoQ supplementation.

22.6 CONCLUSIONS AND FUTURE PROSPECTS

Should the reader feel that the above discussion has led to few definite conclusions regarding whether there is a role for CoQ in alcoholic liver disease, our objective has been achieved. As alcoholic-liver disease involves, at least in part, free radicals in its pathogenesis, and CoQ can be consumed in free radical producing systems,[77] it is possible that ethanol could cause a decrease in hepatic mitochondrial CoQ levels. This could cause a direct decrease in the rate of mitochondrial β-oxidation or have a secondary effect by exacerbating free radical attack in the liver. As there is an interaction between vitamin E and CoQ[78,79] as lipophilic antioxidants and CoQ can regenerate or spare vitamin E both *in vitro*[78] and *in vivo*,[73,80] supplementation with CoQ and/or vitamin E is of possible interest in the amelioration of alcoholic liver disease.

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23 Ubiquinone, Oxidative Stress, and Liver Carcinogenesis

Per Stål and Jerker M. Olsson

CONTENTS

23.1 CARCINOGENESIS IS A MULTISTEP PROCEDURE

The carcinogenic process is a multistep procedure, in which cells acquire properties of the malignant phenotype in a step-by-step manner. This phenotype is characterized by increased resistance to toxic compounds, enhanced cell turnover, uncontrolled growth, and capability of invasion and metastasis.[1] The stepwise development of such changes can be summarized sequentially into three phases: initiation, promotion, and progression[2] (Figure 23.1). The first phase, initiation, involves a mutagenic event leading to a permanent genetic alteration. During the second phase, promotion, the initiated cells are stimulated to proliferate by a promoter, forming clonal cell expansions referred to as preneoplastic foci, or nodules. In the final step of the carcinogenic process, progression, preneoplastic cells aquire genomic instability and unregulated growth, with time leading to the neoplastic phenotype. Additional spontaneous mutations in growth regulatory genes of the labile DNA in these cells lead to the gradual development of a more malignant phenotype, eventually manifested in the malignant tumor.[3]

23.2 THE ROLE OF OXIDATIVE STRESS IN LIVER CARCINOGENESIS

Oxidative stress has been suggested as playing an important role in the propagation of the carcinogenic process.[4–7] Firstly, reactive oxidative species in proximity to the cell nucleus may induce

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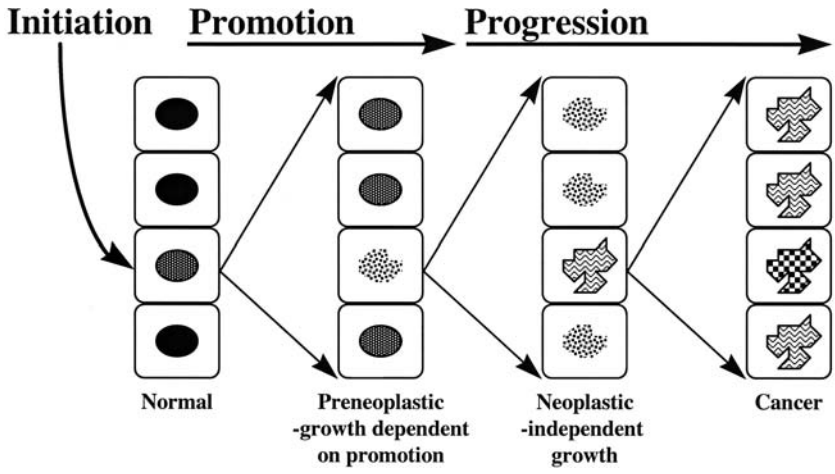

FIGURE 23.1 Hepatocarcinogenesis can be regarded as a multistep procedure in which cells acquire a neoplastic phenotypic expression in a step-by-step manner. The three major phases in this process, initiation, promotion and progression, are outlined in the text.

oxidative DNA-damage of possible importance for initiation.[8–11] Secondly, enhanced oxidative stress may have promotive properties, thus stimulating the proliferation of preneoplastic cells.[4,8,9,12] Thirdly, oxidative damage may play a role during progression by further damaging the unstable genetic material or altering the growth of neoplastic cells.[5–7,13] Thus, the extent of oxidative stress and cellular defense mechanisms, such as antioxidative protection against free radicals, possibly influence the speed at which a normal cell travels toward the neoplastic phenotype. For instance, malignant cells may alter their antioxidant defense mechanisms in order to maintain a high level of resistance to toxic compounds and a high proliferative activity.[3]

In humans, the majority of hepatocellular carcinomas arise in cirrhotic livers. Liver cirrhosis may be considered the end result of long-term damage, often of low-grade severity, and be regarded as a potentially precancerous condition. The etiologies of cirrhosis vary from chronic viral hepatitis infection, autoimmune disorders, toxins like ethanol or iron overload, or chemical carcinogens. In both virus-associated, ethanol-, iron-, or carbon tetrachloride-induced liver cirrhosis, enhanced oxidative stress has been implicated as a major causative factor.[8,14–19] In these patients, antioxidant defense has been shown to be decreased, of possible importance for the propagation of fibrogenesis and subsequently carcinogenesis.[20–23] Thus, besides a possible direct effect on cancer development, oxidative stress may also indirectly influence hepatocarcinogenesis by enhancing the progression of the cirrhotic process.

23.3 IRON OVERLOAD DEPLETES HEPATIC ANTIOXIDANTS

Iron has been used as a model substance in the studies of oxidative liver damage, since iron is known to increase the production of free radicals and enhance oxidative stress.[24,25] In humans, the most common diseases with iron overload are genetic hemochromatosis and transfusional iron overload. Genetic hemochromatosis is an inherited disorder of iron metabolism in which excess iron is absorbed via the gut and deposited in parenchymal organs, predominantly the liver.[26,27] With time, deposition of intracellular iron in hepatocytes results in lipid peroxidation of cellular membranes, impairment of mitochondrial functions, leakage of lysosomal enzymes, and finally iron-induced necrosis (called sideronecrosis).[26] Once sideronecrosis has occurred, Kupffer cells and lipocytes will become activated and collagen synthesis increased,[28] eventually leading to fibrosis, cirrhosis, and an increased risk for the development of hepatocellular carcinoma.[29–31]

Feeding rats with dietary carbonyl iron will mimic the iron-loading pattern of the human disease, and is used as an animal model of precirrhotic hemochromatosis.[32–34] In this model, iron was shown to enhance lipid peroxidation (determined as thiobarbituric acid reactive products)[35,36] and deplete the hepatic contents of α-tocopherol,[35] reduced ubiquinone-9[37,38] and ubiquinone-10.[39] In another animal model using the ferrocene iron-loaded rat, breath ethane exhalation, as a marker of lipid peroxidation, was increased and dependent on the extent of iron overload.[40] Plasma as well as hepatic α-tocopherol decreased with progressive iron loading and a significant depletion in hepatic ubiquinol-9 and -10 was noted.[40]

However, in spite of increased oxygen free radical production in iron-loaded livers, neither cirrhosis[32,33] nor hepatocellular carcinoma[34] have been encountered in animal models of dietary iron overload. In this respect, dietary iron overload may differ from parenterally administered iron dextran, the latter of which was found to act as a promoter in hepatocarcinogenesis in conjunction with diethylnitrosamine (DEN).[41]

In genetic hemochromatosis, the increased cancer risk does not persist if excess iron is removed before the development of cirrhosis,[31] indicating that the cirrhosis may be a greater risk factor for developing hepatocellular carcinoma (HCC) than the iron overload per se. However, additional clinical data suggest that iron-induced oxidative stress may play a role in hepatocarcinogenesis apart from being an inducer of cirrhosis. In one study, livers with and without HCC were compared regarding their iron contents.[19] Livers with HCC had increased iron deposits in the tissue surrounding the tumor, as compared with normal or cirrhotic livers without HCC, or livers with metastatic tumors, suggesting that long-term iron-induced oxidative stress may increase the risk for malignant transformation.

23.4 IRON POTENTIATES ETHANOL-INDUCED OXIDATIVE STRESS

Iron may also act as a cofactor that increases the oxidative liver damage exerted by other hepatotoxins such as alcohol. Ethanol is metabolized by the enzyme cytochrome P4502E1 (CYP2E1). During this metabolism the CYP2E1 generates free radicals,[42] and increased oxidative stress was found in livers exposed to ethanol.[43] When iron is added to ethanol treatment, liver damage and indices of oxidative stress are greatly enhanced.[44,45] We found that the combination of iron and ethanol significantly decreased the hepatic contents of reduced ubiquinone-9 and increased the serum aminotransferase activities, as compared with either substance given alone[38] (Figure 23.2). In this respect, the free iron pool may play a major role in catalyzing oxidative damage exerted by ethanol.[24] In the Fenton reaction, ferrous iron catalyzes the production of hydroxyl radicals from hydrogen peroxide. Indeed, chelating free iron by desferrioxamine reversed the increased levels of aminotransferases that were induced by iron plus ethanol, although the total hepatic iron content was unchanged.[36]

23.5 CARBON TETRACHLORIDE MAY ALTER THE HEPATIC CONTENTS OF ANTIOXIDANTS

Chronic CCl_4 administration is known to induce cell necrosis by increased oxidative stress,[46] and repeated injections of CCl_4 initiate the fibrogenic process in the liver and may result in cirrhosis.[47] Administration of vitamin E prevents CCl_4-induced liver necrosis and cirrhosis, which supports the role of reactive oxygen species in liver damage associated with CCl_4.[48] CCl_4 is metabolically activated by cytochrome P450 to form $^{\bullet}CCl_3$ free radicals, which initiate lipid peroxidation in the cell and decrease cellular ubiquinol-10.[49] Kishi et al. demonstrated that administration of ubiquinone supplement attenuated CCl_4-induced cell necrosis.[49] In a study in which rats were exposed to diethylnitrosamine (DEN) as initiator, followed by repeated injections of carbon tetrachloride (CCl_4)

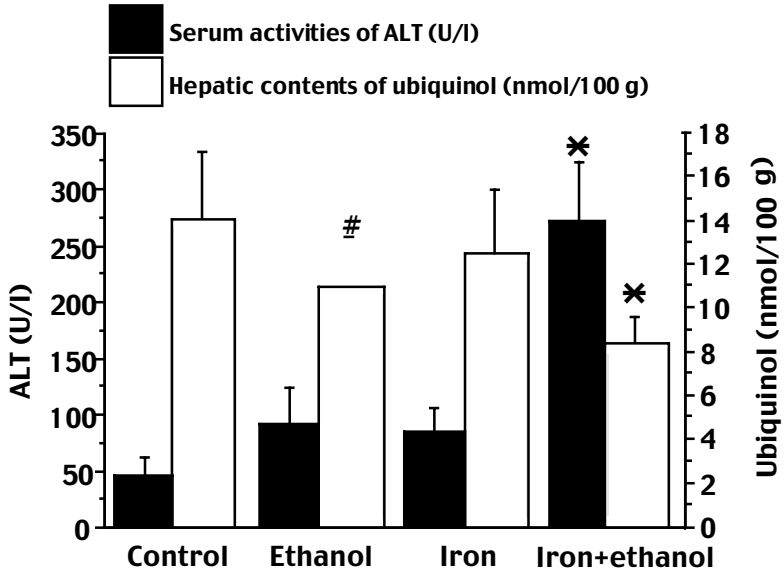

FIGURE 23.2 Serum alanine aminotransferase activities (ALT) and hepatic contents of ubiquinol (reduced ubiquinone-9) in male Wistar rats exposed to dietary carbonyl iron with or without chronic ethanol administration. Animals receiving iron + ethanol demonstrated significantly increased ALT activities in serum compared with the other groups (* = $p < 0.01$). Hepatic contents of ubiquinol were significantly decreased in animals receiving ethanol alone, compared with controls (# = $p < 0.05$), whereas treatment with iron plus ethanol led to significantly decreased levels compared with all other groups (* = $p < 0.05$). (Data from [38]).

to induce liver cirrhosis, we observed interesting changes of antioxidant levels in the livers exposed to this combination. Fourty-eight hours after the first injection of CCl_4 there was a significant increase of hepatic α-tocopherol contents, indicating compensation of the injured liver to oxidative stress, whereas levels of ubiquinone-9 were unchanged. However, both the reduced and oxidized forms of ubiquinone-9 were increased almost threefold after 23 weeks of repeated CCl_4 administration once a week (plus one single injection of DEN at week 8), as compared with the controls receiving DEN alone. These changes of hepatic ubiquinone content possibly reflect an adaptation of the liver to the toxic chemicals, and is the first finding of an induction of ubiquinol synthesis by chronic CCl_4 administration.

23.6 HEPATITIS INDUCES OXIDATIVE DNA DAMAGE OF IMPORTANCE FOR HEPATOCARCINOGENESIS

The necroinflammatory reaction seen in livers with viral hepatitis is the result of a host immune response to viral antigens.[14] Recruited leukocytes and macrophages release reactive oxygen species in areas of infection, which create necrosis of target hepatocytes. The enhanced oxidative stress overwhelms antioxidant defense mechanisms and leads to damage of crucial macromolecules, including DNA. 8-oxo-2′-deoxyguanosine (8-oxo-dG) is continuously produced in liver tissue but in the case of a normal liver, is quickly removed by DNA repair enzymes. However, in chronic hepatitis, oxidative stress is enhanced and cell proliferation increased, which enables DNA replication to occur before the repair of genetic lesions is complete, resulting in mutations. This concept has been tested in the hepatitis B-virus (HBV) transgenic mouse animal model. These mice overexpress the HBV large envelope protein, leading to necrosis, inflammation, and subsequent development of HCC.[12] A significant accumulation of 8-oxo-dG was found in areas with pronounced

inflammation and liver cell proliferation, and the levels of 8-oxo-dG correlated to the extent of the liver disease.[8]

Thus, experimental data strongly indicate that increased oxidative stress is an important pathogenic mechanism in hepatitis-induced liver damage, and these results are supported by clinical data on humans with hepatitis B and C. In these patients, plasma levels of vitamin E were decreased as compared with that of healthy controls, indicating depletion of antioxidants secondary to the hepatitis infection.[21,23] Likewise, in an assay using the plasma ratio of oxidized and reduced ubiquinone as a marker of oxidative stress,[20] patients with hepatitis were found to have a significantly increased ratio as compared with healthy controls.[22] In a prospective randomized double-blind crossover study, patients with chronic hepatitis C refractory to alpha-interferon therapy were treated with high doses of vitamin E, which significantly reduced the serum activities of aminotransferases, indicating a reduction of the liver damage.[50] These findings indicate that enhanced free radical production may be of importance in the pathogenesis of virus-associated liver cancer development.

23.7 INTERACTION BETWEEN IRON, ALCOHOL, AND HEPATOTROPIC VIRUSES

Theoretically, in livers with viral hepatitis displaying chronic necroinflammation and enhanced leukocyte production of superoxide and hydrogen peroxide, iron in excess could catalyze production of hydroxyl radicals and increase the damaging effect of the viral infection. This hypothesis has not yet been proven *in vivo*, but clinical findings point to a potential synergism between iron and the chronic virus infection. High liver iron contents decreased the response rate to alpha-interferon in chronic hepatitis C.[51–53] In successful cases, liver iron content decreased following treatment with alpha-interferon,[54] which was interpreted as secondary to reduced inflammation, as well as a factor influencing the improvement in liver histology. In line with these results are data demonstrating a beneficial effect on serum aminotransferase levels in hepatitis C patients treated with phlebotomies to remove excess iron, although clearance of the virus was not affected.[55]

Similarily, clinical data suggest an additive effect of ethanol and chronic viral hepatitis, the combination of which enhances oxidative cell damage and leads to a more severe liver disease.[56] In patients with hepatitis C and hepatocellular carcinoma, a high alcohol intake was related to increased tumor growth.[17] Chronic exposition to alcohol alters cell organelle membranes, especially in the endoplasmic reticulum, which may be of relevance for the development of neoplasia.[15] In addition, ethanol induces liver enzymes which may be of importance in metabolism of carcinogenic compounds, and ethanol could therefore modulate one or more steps of the carcinogenic process.[18]

These findings indicate that both iron- and ethanol-induced free radical production may enhance the virus-associated liver damage and cancer development, although the exact roles of these hepatotoxic compounds in virus-associated hepatocarcinogensis still has to be determined.

23.8 THE SOLT AND FARBER ANIMAL MODEL OF CHEMICAL HEPATOCARCINOGENESIS

When studying the effects of xenobiotics and antioxidants during liver carcinogenesis, experimental models have been created. One of the most commonly employed models for this purpose is the Solt and Farber model of chemical hepatocarcinogenesis,[57] in which the effects of carcinogens or anticancer drugs can be studied sequentially during initiation, promotion, and progression respectively (Figure 23.3).[53] In this animal model on rat liver, commonly known as the "resistant hepatocyte model,"[3] initiation is accomplished by diethylnitrosamine (DEN), a genotoxic compound that causes extensive DNA damage. To induce cell proliferation before DNA repair is complete, DEN is administered in a high, necrogenic dose, which leads to a burst of regenerative cell division and fixation of the DNA damage, or DEN is combined with partial hepatectomy. After this treatment,

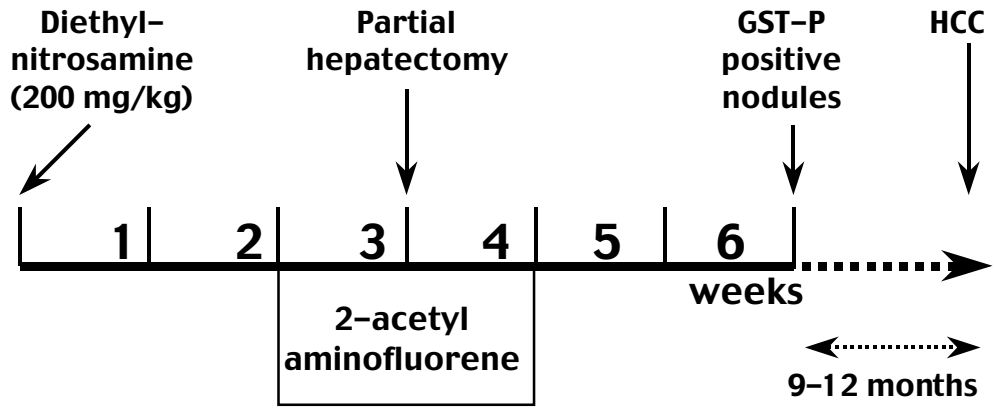

FIGURE 23.3 Schematic presentation of the Solt and Farber model of chemical hepatocarcinogenesis. Initiation is performed by administration of a necrogenic dose of diethylnitrosamine (DEN). Two weeks later, promotion is started by dietary supplementation of 0.2% 2-acetyl aminofluorene (2-AAF) for two weeks. 2-AAF inhibits proliferation of noninitiated cells, and to stimulate division of the initiated cell compartment, a two-third partial hepatectomy is performed. Two weeks after the cessation of promotion, preneoplastic nodules can be visualized in the liver by immunohistochemical techniques (with antibodies towards glutathione S-transferase P). Nine to twelve months later, hepatocellular cacinomas have developed in all livers exposed to this regimen. GST-P = glutathione S-transferase P; HCC = hepatocellular carcinoma.

single cells expressing the initiated phenotype are scattered throughout the liver.[1] The initiated phenotype includes increased cellular levels of glutathione S-transferase P (GST-P), which can be visualized by immunohistochemistry.[3]

Initiated cells express a growth advantage, with increased responsiveness to proliferative stimuli, and/or increased resistance to toxic compounds.[2,59] These properties are utilized during promotion, which is accomplished by a proliferative stimulus (partial hepatectomy or carbon tetrachloride-induced cell necrosis). In order to inhibit proliferation of noninitiated cells, a mitoinhibitory compound (2-acetyl aminofluorene, 2-AAF) is administered simultaneously with the proliferative stimulus.[57] 2-AAF blocks cell division of normal cells, but initiated cells are resistant to the mitoinhibitory effects of 2-AAF. They expand into clones known as preneoplastic foci or nodules. A fraction of foci will spontaneously remodel into normal cells, whereas persistent foci acquire additional genetic alterations leading to independent growth. Persistent foci have a genetic instability, and during the third step, progression, multiple mutations of the labile DNA affects growth regulatory genes or tumor suppressor genes, subsequently leading to a more malignant phenotype with uncontrolled growth. As for the Solt and Farber model of hepatocarcinogenesis, all treated animals eventually develop HCCs in 9 to 12 months time.[3]

23.9 OXIDATIVE STRESS DURING INITIATION AND PROMOTION

The animal model described above has been widely used as an *in vivo* assay to study the effects of various actions or xenobiotics on liver carcinogenesis.[58] Using this model, the number of foci was shown to correspond to the initiating potency, whereas their relative volume was related to the strength of the promoter. Dietary carbonyl iron, known to induce the formation of reactive oxygen species[25,35] and decrease hepatic levels of antioxidants,[37,39] did not act as initiator or as promoter in this model.[34] Carbonyl iron causes decreased weight gain of animals and has mitostimulatory properties on surrounding, noninitiated hepatocytes, both of which decrease the formation of preneoplastic nodules.[34] Promotive effects of oxidative stress were seen in experiments in which

free radical formation was induced by redox-modulation,[6] parenteral iron dextran,[41] or repeated injections of carbon tetrachloride.[37]

23.10 ROLES OF OXIDATIVE STRESS AND UBIQUINONE DURING TUMOR PROGRESSION

The impact of oxidative stress during progression of preneoplastic foci into HCCs has been studied during hepatocarcinogenesis.[60,61] We evaluated the effects of long-term exposure to dietary iron during the progression step, and measured tumor contents of antioxidants.[61] Although HCCs were histologically iron-deficient, exposure to dietary iron depleted their contents of reduced ubiquinone-9. This finding is in line with results showing an increased uptake of free iron in neoplastic liver cells as compared with nonneoplastic hepatocytes.[62] The demonstration of decreased levels of antioxidants and increased contents of iron and ferritin in cultured hepatoma cells supports this hypothesis.[63] In spite of the reduced levels of ubiquinone-9 in HCCs, the number and size of carcinomas were similar between groups, and concentrations of α-tocopherol in tumors were not altered by iron. On the contrary, tumors from iron-treated rats had a higher differentiation as compared with those from control animals. Thus, iron-induced oxidative stress does not enhance the progression of HCCs during liver cancer development.

Ubiquinones function as a defense against the initiation of lipid peroxidation, while α-tocopherol inhibits further propagation of this process.[64] We found that levels of α-tocopherol in HCCs were unaltered by treatment with iron, which contrasts with the depletion of vitamin E seen in normal liver after feeding with carbonyl iron.[39] Therefore, the decreased levels of ubiquinone but unaltered contents of vitamin E in HCCs exposed to iron may be interpreted to mean that tumor cells utilize ubiquinone as their primary defense against iron-induced oxidative stress, in which case the oxidation of ubiquinones would protect against further depletion of α-tocopherol.

These results are supported by data from Denda et al., who used redox-enzyme modulation with phenobarbital to increase cytochrome P450 reductase, phorone to deplete glutathione, dicumarol to inhibit DT-diaphorase, and had iron as a supplement.[6] This regimen enhanced oxidative stress and acted as promoter in liver carcinogenesis. However, the treatment neither acted as initiator nor progressor when given for 33 weeks to nodule-bearing rats induced by DEN. On the contrary, the number of hepatocellular carcinomas developing in nodule-bearing rats was significantly reduced by redox-enzyme modulation treatment.[60] These results support the concept that preneoplastic nodules are resistant to oxidative stress during progression.

23.11 CONTENTS OF UBIQUINONE ARE INCREASED IN PRENEOPLASTIC HEPATIC NODULES

One reason why preneoplastic liver cells are more resistant to oxidative stress may be increased intracellular levels of antioxidants.[3] To study concentrations and synthesis of antioxidants in preneoplastic foci, an animal model developed by Epstein et al.[65] and modified by Eriksson et al.[66] was used. In this model, rats were treated with 0.05% dietary 2-acetylaminofluorene (2-AAF) for 1 to 3 weeks, followed by a control diet for 1 to 2 weeks, in an intermittent regimen during 25 weeks. Ten weeks later, large preneoplastic nodules developed throughout the liver parenchyma, occupying 50 to 80% of the liver volume. These nodules can be dissected and preneoplastic cells harvested in sufficient amounts suitable for subcellular fractionation.

We have performed investigations on ubiquinone concentrations and synthesis during hepatocarcinogenesis chemically induced by this model.[67,68] The concentrations of ubiquinone in the microsomal fractions (containing membranes of the Golgi apparatus) and lysosomal fractions from preneoplastic cells were increased six- and two-fold, respectively, compared to control liver tissue, while the concentration in mitochondria was unchanged.[67] The elevated concentrations of ubiquinone

in extramitochondrial compartments was suggested to be an attempt of the resistant preneoplastic cell to prevent free radical damage by increasing the synthesis of ubiquinone. This proposal is supported by the fact that preneoplastic cells are selected during the carcinogenic process by their resistance to 2-AAF, whereas normal hepatocytes are mitoinhibited by the same drug.

Furthermore, we showed that several enzyme activities of the mevalonate pathway, which are of importance for the synthesis of ubiquinone, were elevated in preneoplastic cells compared to control hepatocytes.[68] For instance, the enzymatic activities of HMG-CoA reductase, farnesyl pyrophosphate synthase, and nonaprenyl-4-hydroxybenzoate transferase were increased four-, two-, and two-fold, respectively. The enzyme nonaprenyl-4-hydroxybenzoate condenses the trans-polyprenyl pyrophosphate side-chain to the precursor ring 4-hydroxybezoate, which enzymatically is converted in several steps to the final structure of ubiquinone.[69] These results may explain some of the six-fold increased concentration of ubiquinone in the microsomal fraction, since synthesis of the side-chain occurs in the endoplasmic reticulum, and the condensation of this chain to the precursor ring takes place in the Golgi apparatus.[70]

Recently we found that the enzyme lipoamide dehydrogenase efficiently reduces ubiquinone to its antioxidative active form ubiquinol.[71] This enzyme is traditionally known to be one of three enzymes in the α-ketoacid dehydrogenase complex that oxidizes pyruvate, α-ketoglutarate, and the branched-chain α-ketoacids. Lipoamide dehydrogenase is present at the matrix surface of the inner mitochondrial membrane in all eukaryotic organisms studied to date. However, this enzyme has also been found in increased amounts in cytosol of preneoplastic rat liver cells (unpublished data). As this enzyme can regenerate ubiquinone to its antioxidative active form ubiquinol, the elevated levels of lipoamide dehydrogenase may enhance reduction of ubiquinone and prolong its half-life, contributing to the increased amounts of ubiquinone determined in preneoplastic cells.

23.12 CONTENTS OF UBIQUINONE ARE DECREASED IN HEPATOCELLULAR CARCINOMAS

In contrast to the elevated levels found in preneoplastic liver nodules, fully developed HCCs often display reduced amounts of ubiquinone. Unpublished data from our research group show that the concentration of ubiquinone continuously changes during the hepatocarcinogenic process, being elevated in preneoplastic foci and decreased in neoplastic cells, and that the levels partly correspond to the phenotypic differentiation grade of the tumor. Similarily, in human HCCs it was found that the concentration of ubiquinone was decreased to half of the level of control liver tissue.[72] In these studies microsomes from normal human liver tissue and HCCs were isolated and the activity of the main regulatory enzyme of the mevalonate pathway, HMG-CoA reductase, was analysed.[72,73] Microsomes from HCCs demonstrated a doubling of HMG-CoA reductase activity compared to those from normal liver tissue. Although data from the purification of microsomal fractions from HCCs may be difficult to interpret due to heterogeneity of the tumors, these results are interesting in the context of mevalonate pathway regulation.

To estimate the synthesis rates of cholesterol, ubiquinone, and dolichol in homogenates from human control liver and HCCs, radioactively labeled mevalonic acid was used in *in vitro* incubations. These analyses demonstrated an almost 50% decreased synthesis of ubiquinone in HCCs compared with control tissue, whereas cholesterol synthesis was doubled, and the dolichol synthesis was only 10% of that found in control livers.[72,73] These results suggest that the synthesis of those lipids are regulated independently in HCCs subsequent to the branch point of farnesyl pyrophosphate.

The concentrations of ubiquinone in surgical biopsy materials from highly differentiated human HCCs were found to be reduced to half the levels of normal liver tissue derived from patients without HCC.[74] These results were reproduced on material from HCCs obtained shortly after death or directly after surgery.[75] Also, the phenotypic differentiation of the HCCs was studied in relation to tumor contents of ubiquinone, but no correlation between these two variables could be found.[76]

23.13 EFFECTS OF DIETARY SUPPLEMENTATION OF UBIQUINONE ON CARCINOGENESIS

The results showing reduced levels of ubiquinone in HCCs are thus in concordance with data from experimental animal models and on human cancers. Increased levels of ubiquinone in preneoplastic foci may contribute to their increased resistance to oxidative stress. However, the reasons as to why these cells decrease their levels of ubiquinone during malignant transformation remain unclear. In light of these findings, reports of ubiquinone levels in blood from cancer patients is pertinent. Folkers et al. reported decreased blood levels of coenzyme Q10 in patients with myeloma and breast cancer.[77] In this study, the fractions of cancer patients with blood levels of coenzyme Q10 below 0.5 μg/ml (breast cancer) and 0.7 μg/ml (myeloma patients) were significantly increased compared with ordinary people. However, 49% of the ordinary people had levels below 0.8 μg/ml, indicating a wide range of values in all groups studied. Only myeloma patients had significantly reduced mean blood levels of coenzyme Q10 as compared with ordinary people.[77] Reports studying the effects of dietary supplementation with nutritional antioxidants, essential fatty acids, and coenzyme Q10 on patients with "high-risk" breast cancer indicate a tumor-suppressive effect by this regimen.[78] However, these findings are not supported by other studies in which two months of supplementation with antioxidants, including ubiquinone-10, did not result in significant changes in the urinary excretion rate of 8-oxo-dG, although the plasma antioxidant concentrations were increased.[79]

23.14 FOOD RESTRICTION INCREASES UBIQUINONE IN NONNEOPLASTIC AND NEOPLASTIC LIVER TISSUE

Diet restriction has been known to exert an inhibitory effect on liver carcinogenesis. The pathogenic mechanisms for this effect are not completely elucidated, but increased apoptosis, decreased cell division, and decreased oxidative stress in livers exposed to caloric restriction have been suggested.[80] In a study on antioxidant levels in normal liver tissue from animals exposed to diet restriction, Willis et al. found enhanced levels of coenzyme Q as compared with that found in livers from animals fed ad libitum.[81]

We asked if the contents of ubiquinone-9 and -10 would increase in HCCs from rats exposed to food restriction during progression in the Solt and Farber protocol of chemical hepatocarcinogenesis.[82] A long-term, moderate food restriction during tumor progression significantly reduced the number of HCCs. Interestingly, carcinomas from food-restricted rats had increased contents of reduced and oxidized ubiquinone-9 and -10. These results indicate enhanced protection from oxidative damage in tumors exposed to diet restriction. However, the ratio between the reduced and oxidized forms of ubiquinone was unchanged in tumors from food-restricted rats. Hence, one may speculate that synthesis and/or half-life of ubiquinone[83] is increased in HCCs exposed to food restriction, maybe by the induction of ubiquinone-regenerating enzyme systems.[71,84] Thus, as in the case of normal liver,[80,81] caloric restriction modulates the sensitivity to oxidative stress and increases the levels of ubiquinone-9 and -10 in HCCs.

In the Solt and Farber model, food restriction during progression stimulated cell division and apoptosis of HCCs (Wang et al., submitted). Ubiquinone was reported to stimulate the growth of cultured HeLa and Balb/3T3 cells in serum-free conditions, possibly by stimulating the plasma membrane NADH oxidation or by modifying the membrane quinone redox balance.[85] Ubiquinone is an essential factor required in the electron transport system of the hepatocyte in rat, and if it was extracted from the membrane, the activities of NADH dehydrogenase and NADH: oxygen oxidoreductase decreased.[86] However, there was no significant correlation between cell proliferation and contents of ubiquinone in HCCs from rats exposed to food restriction.[82] Thus, a putative role of ubiquinone in the regulation of cell proliferation of HCCs *in vivo* remains to be proven.

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Section 3D

Physical Performance/Training

24 Effects of Ubiquinone-10 Supplementation on Physical Performance in Humans

Christer Malm and Michael Svensson

CONTENTS

24.1 INTRODUCTION

Physical exercise induces various types of stress in the body. The effects of physical stress on an organism depend on a number of different factors, including previous exposure to stress, genotypic adaptation capacity, nutritional status, and immune system functions. Response to a given bout of exercise or training regime will thus vary significantly not only between individuals, but also in the same subject. In a complex organism like a mammal, adaptation to physical training is therefore a delicately orchestrated series of events controlled by numerous factors. Some of these factors are well known but most are yet to be discovered. Over the past few decades many attempts have been made to optimize adaptation to training and increase physical performance by improving equipment, nutrition, training program, and other related factors.

A growing number of studies suggest that physical exercise is associated with increased production of reactive oxygen species (ROS) and an alteration in the antioxidant defense systems. Some of these alterations are associated with tissue damage (for review, see Sen, K. et al.[1]). However, recent research indicates that ROS have important functions as signal transduction molecules and are involved in regulation of gene expression and enzyme activity.[2] Therefore, ROS are vital for cellular homeostasis, development, and adaptation. Alteration in the balance between pro- and antioxidant systems could either enhance or reduce cell function and adaptation capacity.

Several studies have shown exercise-induced changes in antioxidant systems in plasma and skeletal muscle in humans.[1] In general, the conclusion in many of these studies has been that a decreased "defense" against ROS will have negative consequences in terms of increased tissue damage and decreased cell function and adaptation to stress. In the context of this chapter, the ultimate result is impaired physical performance. In order to prevent these hypothetically negative (side) effects of physical exercise, supplementation with different types of antioxidants has been used in a number of studies.[3–10]

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Because of the multifunctional role of Q_{10} (ubiquinone-10, coenzyme Q_{10}), including antioxidant properties and electron transferring functions in various cell membranes, great attention has been focused on weather tissue Q_{10} levels could be a limiting factor during strenuous physical exercise. It has been speculated that an increased ROS production during physical exercise could decrease Q_{10} level in muscle tissue and negatively affect physical performance,[11] at least in subjects undertaking strenuous physical training. Supplementation with Q_{10} could therefore, hypothetically, "normalize" or even enhance physical performance. Several criteria, including uptake of ingested Q_{10} in blood and muscle tissue, as well as functional incorporation in mitochondrial and cell membranes, must be fulfilled before Q_{10} supplementation will enhance cellular function and physical performance according to these hypotheses. Also, a distinction between the effects of Q_{10} as an antioxidant and as an electron carrier should be addressed. Different investigators have consequently tested several related hypotheses regarding Q_{10} and physical performance.[3–10,12–14]

To our knowledge, no study to date has demonstrated a direct link between physical performance and alteration in antioxidant capacity (including Q_{10}) in blood or muscle tissue in humans. Some studies have shown a correlation between physical performance and Q_{10} concentration in skeletal muscle.[11,15] However, because Q_{10} is mostly localized in mitochondrial membranes, this correlation is due to the mitochondrial density in skeletal muscle tissue. Mitochondrial density depends on muscle fiber type and physical training status as well as genetic factors. Published results do not support the concept that oral Q_{10} supplementation will increase Q_{10} concentration in healthy human muscle tissue, or that increased muscle or plasma Q_{10} concentration per se is related to increased physical performance.[16]

Nevertheless, because of the multiple functions of Q_{10} (discussed in detail elsewhere in this book) and speculative hypotheses, Q_{10} has emerged as one of the most popular antioxidant supplements among elite athletes in various sports, as well as others involved in recreational physical activities.

The main objective of this chapter is to present and discuss results obtained in a number of experimental studies where Q_{10} supplementation and physical performance has been investigated.

24.2 RESULTS

Many researchers have hypothesized that supplementation with ubiquinone-10 (Q_{10}) may enhance athletic performance.[3,6,17,18] Based on current knowledge regarding the function of Q_{10} in human muscle cells, these are valid theoretical speculations. Some *in vitro* data can also be interpreted in favor of beneficial effects on cell function of increased Q_{10} concentrations in working muscle cells.[19,20] Caution must always be taken when extrapolating *in vitro* data to *in vivo* situations. For example, direct intravenous injection with high Q_{10} concentration (10 mg/kg) in isolated rabbit heart in the study by Takeo et al.,[20] or direct administration of Q_{10} in cultured rat muscle cells followed by electric stimulation[19] might not be of physiological relevance. In these situations, Q_{10} is administered directly into the muscle tissue and concerns regarding physiological uptake and availability are avoided. The majority of the data so far published demonstrates no positive effects of oral Q_{10} supplementation on physical performance in healthy subjects.[3,5,7,9,13] On the contrary, one study with strenuous anaerobic training for only five days demonstrated reduced physical performance improvement compared to a placebo-control group.[6] Furthermore, longer cycling time to exhaustion in the placebo group compared to the Q_{10} supplemented group was recorded in a study by Laaksonen et al.[5] Several studies claim increased physical performance with Q_{10} supplementation.[4,10,12,18,21] However, these studies all have questionable designs or statistical interpretations of data (Table 24.1). Many of them lack a placebo-control group,[4,10,12,14,17,18,21] have used other nutritional supplements in combination with Q_{10},[5,7,8,17,20] or fail in the statistical analysis.[4,12,17,18,21] None of these studies have controlled physical training or controlled diet during the supplementation period.

In subjects with various heart diseases, there may be positive effects with oral Q_{10} supplementation on physical performance. Few placebo-controlled studies have been performed to investigate this possibility.[17,22–25]

TABLE 24.1
Summary of Articles Published on Ubiquinone-10 Supplementation and Physical Performance. Abbreviations: Lac = Lactate, HR = Heart Rate, WL = Work Load, SV = Stroke Volume, $VO_{2\,max}$ = Maximal Oxygen Consumption, d = days, w = weeks, m = months, N.a. = Not Applicable

Reference	Training	Testing	Dose Q_{10}	Variables	Time	Placebo	Subjects	Effects of Q_{10}	Comments
Aerobic Exercise									
Braun, B., et al. 1991[3]	Cycling	Cycling (submax and max)	100 mg/day	Respiratory capacity and work (KJ)	60 d	Yes	Male Elite cyclists	None	Training volume reported by subjects
Laaksonen, R., et al. 1995[15]	Unknown	Cycling (max)	120 mg/day	Time to exhaustion, $VO_{2\,max}$	6 w	Yes	Young and older male	↑ Time to exhaustion in placebo	No testing before treatment. ω-3 fatty acid also supplemented
Nielsen, A. N., et al. 1999[7]	Triathlon	Cycling (max)	100 mg/day	Respiratory capacity and muscle metabolism	12 w	Yes	Male Triathletes	None	Vit C and $\propto$-tocopherol also supplemented Training not controlled
Porter, D. A., et al. 1995[13]	None	Handgrip, cycling (max)	150 mg/day	VO_2, Lac, HR, WL	2 m	Yes	Male Middle aged Untrained	None	Training not controlled
Snider, I. P., et al. 1992[8]	None	Running and cycling (max)	100 mg/day	Running speed, respiratory capacity	4 w	Yes	Male Triathletes	None	Vit C, vit E cytochrome C and inosine also supplemented; Cross-over design; Training not controlled;
Weston, S. B., et al. 1997[9]	Cycling and Triathlon	Cycling (submax and max)	1 mg/kg/day	Respiratory capacity and blood metabolites	28 d	Yes	Male Elite cyclists Triathlets	None	Training not controlled
Ylikoski, T., et al., 1997[10]	Unknown	Pole-walking (submax and max) Protocol unclear	90 mg/day	Respiratory capacity	6 w	Yes	Male	↑ $VO_{2\,max}$, aerobic and anaerobic threshold	Author and Q_{10} supplier the same; Exercise testing protocol unclear; $VO_{2\,max}$ weight-related; Unclear statistics; Training not controlled

(Continued)

TABLE 24.1 (Cont'd)
Summary of Articles Published on Ubiquinone-10 Supplementation and Physical Performance. Abbreviations: Lac = Lactate, HR = Heart Rate, WL = Work Load, SV = Stroke Volume, $VO_{2\ max}$ = Maximal Oxygen Consumption, d = days, w = weeks, m = months, N.a. = Not Applicable

Reference	Training	Testing	Dose Q_{10}	Variables	Time	Placebo	Subjects	Effects of Q_{10}	Comments
Zuliani, U., et al. 1989[14]	None	Cycling (submax and max)	100 mg/day	Metabolites	2 m	No	Male Untrained	↓ FFA after exercise	No control group; Training not controlled
				Anaerobic Exercise					
Malm, C., et al. 1997[6]	Cycling	Cycling (submax and max)	120 mg/day	Respiratory capacity anaerobic performance	20 d	Yes	Male	Reduced performance improvement No change in $VO_{2\ max}$	Five days controlled training and testing
				Heart Conditions					
Awata, N., et al. 1980[17]	None	Cycling (max)	30 mg/day	Cardiac function	4–12 w	No	Male with ischemic heart disease	↑ cycling work load ↓ ST depression	No comparison with control, other drugs not restricted, no statistics, unproven diagnostic criteria Training not controlled
Hofman-Bang, C., et al. 1995[22]	None	Cycling (max)	100 mg/day	Ejection fraction, exercise capacity	3 m	Yes	Chronic congestive heart failure	↑ Exercise capacity (Watt)	Cross-over design Training not controlled
Kamikawa, T., et al. 1985[23]	None	Treadmill (submax)	150 mg/day	Respiratory and cardiac function	4 w	Yes	Older male with angina pectoris	↑ Duration, delayed ST-segment change	Training not controlled
Morisco, C., et al. 1994[24]	None	Cycling (max)	150 mg/day	Respiratory and cardiac function	4 w	Yes	Chronic heart failure	↑ Ejection fraction ↑ Cadiac output (HR)	Cross-over design Training not controlled. No effect on exercise tolerance

Permanetter, B., et al. 1992[25]	None	Cycling (max)	100 mg/day	Ejection fraction, end diastolic diameter, cardiac output	4 m	Yes	Idiopathic dilated cardiomyopathy	None	Cross-over design Training not controlled
				In vitro					
Okamoto, T., et al. 1995[19]	N.a.	Electrical stimulation	5 μM	LDH, $[Ca^{2+}]_i$, ATP, lactate	N.a.	N.a.	Rat skeletal muscle culture	↑ Duration ↓ $[Ca^{2+}]_i$	
Takeo, S., et al. 1987[20]	N.a.	Electrical stimulation	10 mg/kg	Contractile force, resting tension, metabolites	N.a.	N.a.	Rabbit heart	↑ Force ↓ Resting tension, perfusion pressure	Vehicle for Q_{10} also changed variables
				Unpublished studies					
Amadio, A., et al. 1991[12]	Basketball	Cycling (submax)	100 mg/day	Cardiac capacity, VO_2	40 d	No	Male Basketball players	↑ Cardiac function (↑ $VO_{2\,max}$?)	No comparison with control, VO_2 only estimated Training not controlled
Fiorella, P.I., et al. 1991[4]	Running	Treadmill (max)	100 mg/day	Running time, distance	40 w	No	Male Runners	(↑ Running time and distance?)	No data or statistics shown; Training not controlled
Vanfraechem, J. H. P., et al. 1981[18]	None	Cycling (max)	60 mg/day	Respiratory and cardiac capacity	4–8 w	No	Male Untrained	↑ VO_2 ↑ SV	No control group No statistical analysis Questionable conclusion Training not controlled
Vanfraechem, J.H.P., et al. 1986[27]	None	Cycling (max)	99 mg/day	Respiratory and cardiac capacity	12 w	Yes (?)	Male with heart failure	↑ VO_2 ↑ SV	Results from placebo group not presented Other drugs not restricted
Wyss, V., et al. 1990[21]	Unknown	Unknown	100 mg/day	Respiratory capacity and blood metabolites	30 d (?)	Yes	Male	↑ $VO_{2\,max}$, max work, lactate	$VO_{2\,max}$ before supplementation not presented for groups Training not controlled

The difficulties in defining as well as measuring physical performance must also be taken into consideration when reviewing published data. In essence, physical performance is related to the activity in question. It is dependent on, and limited by activity-specific factors that may or may not include variables such as $VO_{2\ max}$, muscle strength, local enzyme activity, work efficiency, and psychological factors. An improvement of physical performance of 1% or less, is of significant importance for the athlete but hardly detectable in the laboratory setting. For example, because of methodological limitations as well as day-to-day biological variations among the subjects, the accuracy of repeated measurements of maximal oxygen uptake ($VO_{2\ max}$), a common indicator of aerobic physical performance, is in the range of 3 to 5 %, even with experienced personnel and the most sophisticated equipment used today. Thus, in order to detect a 1% change of this variable, the number of subjects studied needs to be in the 100s.

The limiting factor for $VO_{2\ max}$ during exercise with large muscle groups is circulatory-related and not limited by local muscle capacity.[26] It is therefore questionable that even a functional increase in muscle Q_{10} concentration and/or increased rate of electron transport in mitochondria with Q_{10} supplementation would increase $VO_{2\ max.}$

In contrast to $VO_{2\ max}$, physical performance, also in activities of long duration, is often limited by local muscle capacity. Thus, physical performance may or may not be closely related to $VO_{2\ max}$. It is therefor suggested that in order to investigate proposed changes in physical performance with nutrition supplements, methods other than $VO_{2\ max}$ should be applied.

24.2.1 Aerobic Exercise

The function of Q_{10} as an electron shuttle in the electron transport chain has been suggested as a limiting factor in strenuous endurance exercise where aerobic energy production is of great importance. Consequently, a majority of the studies investigating the effects of Q_{10} supplementation on physical performance have focused on physical performance of longer duration, which by necessity is of mostly aerobic character.[3–5,7–10,12–14,18,21] In summary, no well-designed, peer-reviewed studies show any positive effects on aerobic physical performance, usually measured as $VO_{2\ max}$ or other variables such as performed work, blood lactate, and other blood metabolites, heart rate, or running speed with Q_{10} supplementation (Table 24.1).

Further support for these findings is given by Laaksonen et al.[5] In this cross-over designed, double blind study, 19 subjects (11 young and 8 older) were supplemented with either Q_{10} (120 mg/day) or placebo for 6 weeks. A prolonged maximal cycling test was performed at time of cross-over and after treatment. Interestingly, cycling time to exhaustion was longer in the placebo compared to the Q_{10} group ($p = 0.003$) after treatment in both younger and older subjects. Unfortunately, no exercise performance test was completed before the first supplementation period began, only at the time of cross-over and at end of the study. Subjects (placebo and Q_{10}) also received 500 mg/day of fish oil during the supplementation period. Training volume and intensity, as well as diet and other nutritional supplements were not reported.

In other placebo-controlled studies with well-trained subjects, Q_{10} supplementation alone[3,9] or Q_{10} in combination with vitamins C and E,[7] vitamins C and E, cytochrome *c*, and inosine[8] had no significant effect on respiratory capacity, performed work, or muscle metabolism (P_i/PCr, PCr/ATP, pH). These studies have used cycling and/or running exercise to measure submaximal and maximal physical performance. Unfortunately, only the study by Braun at al.[3] gives $VO_{2\ max}$ in mL/min. Thus, any change in $VO_{2\ max}$ in the other studies may have been disclosed by changes in the subject's body mass.

In untrained subjects, Porter et al.[13] did not find any changes in forearm a-vO_2, blood flow, or lactate release during forearm-hand grip exercise after treatment with Q_{10} for 2 months. Similarly, no change in $VO_{2\ max}$, lactate threshold, heart rate, or maximal workload was noted during maximal cycle ergometry testing. Subject's rating of vigor was reportedly increased in the Q_{10} supplemented group, but was not different than the placebo group.

One study by Ylikoski et al.[10] demonstrates a small (1.6 mL/min/kg) but significant ($p = 0.02$) increase in $VO_{2\ max}$ with 12 weeks of Q_{10} supplementation compared to a placebo control group.

In this study, $VO_{2\,max}$ is reported in mL/min/kg body mass, not mL/min and a change in the subject's body mass could have given the reported increase in $VO_{2\,max}$ in mL/min/kg. Anaerobic and aerobic threshold is also reported to increase, but the incremental testing protocol used is dubious because the workload was increased by 6 mL O_2/kg body mass every 3 min. Treadmill speed and incline is not reported. It is therefore unclear how a change in VO_2 with increased workload could be detected. If VO_2 was increased at the same submaximal workload, this must be interpreted as a decrease in cycling efficiency. Training volume and intensity is also not reported. This should be of great interest because the subjects in this study were elite cross-country skiers who vary their training considerably depending on the time of the year.

In a number of studies presented in edited books, significant improvements in physical performance with Q_{10} supplementation in healthy subjects have been claimed.[4,12,18,21] The results from these studies are difficult to evaluate because of various flaws in protocol designs, statistical analysis, and interpretation of data. Amadio et al.[12] report increased cardiac function and 18% increase in $VO_{2\,max}$ after 40 days of Q_{10} supplementation in basketball players. In this study, no comparison was made with a placebo-controlled group and $VO_{2\,max}$ was only estimated from submaximal ergometer testing. Because training was not controlled and results not compared with the placebo group, conclusion regarding the effects of Q_{10} supplementation is not possible.

Even though Fiorella et al.[4] claim that Q_{10} supplementation increased running performance with 7.9 to 12.9%, no data or statistical analyses to support this claim are presented in the study. Evaluation of the results is therefore impossible. Likewise, conclusions made by Vanfraechem et al.[18] of increased physical performance with Q_{10} supplementation is not supported by presented data. On the contrary, a claimed (no statistics shown) increase (from 36.5 ± 4.8 to 39.4 ± 9.4 mL/kg/min) in VO_2 at a heart rate of 170 beats/min indicates a decreased exercise capacity after supplementation. In another unpublished observation by Wyss et al.[21], conclusions are made in favor of Q_{10} as a performance enhancing supplement. In the design of the study, the posttreatment data for the placebo and Q_{10} groups are compared with combined data from both groups before the study, making any interpretation impossible. It also appears as if well-trained individuals were assigned to the Q_{10} group and the less trained individuals to the placebo-control group.

In conclusion, seven published, placebo-control studies from seven different laboratories demonstrate no effects on aerobic physical performance with Q_{10} supplementation.[3,5,7–9,13,14] One study has noted a small increase in $VO_{2\,max}$[10] but these results must be viewed with care. Four studies from two research groups claim increased aerobic physical performance with Q_{10} supplementation in healthy subjects.[4,12,18,21] However, these studies all have major design, analysis, or methodological uncertainties and are not published in peer-reviewed scientific journals.

With presently published data at hand and considering some unpublished data, it has not been scientifically demonstrated that oral supplementation with ubiquinone-10 (co-enzyme Q_{10}) has any beneficial effects on aerobic physical performance in healthy men.

24.2.2 Anaerobic Exercise

Only one study regarding the effects of Q_{10} supplementation on anaerobic physical performance has been conducted.[6,16] In this placebo-controlled, double blind study, anaerobic as well as aerobic physical performance and adenine nucleotide catabolism was investigated. The study consisted of 22 days of Q_{10} supplementation including 4 days of controlled anaerobic cycling training twice daily from day 11 to day 14. An anaerobic cycling test was performed on days 1, 11, 15, and 20. It was concluded that during strenuous anaerobic training, the increase in physical performance recorded in the placebo group was significantly lower in the Q_{10} supplemented subjects. Notably, even though plasma Q_{10} concentration increased significantly with supplementation, there was no increase in muscle or isolated mitochondria Q_{10} concentration.[16] A negative correlation between changes in Q_{10} concentration in plasma and changes in performance on the anaerobic cycling test, from before to after supplementation was found (Figure 24.1). Thus, increased plasma Q_{10} concentration seems to have resulted in impaired

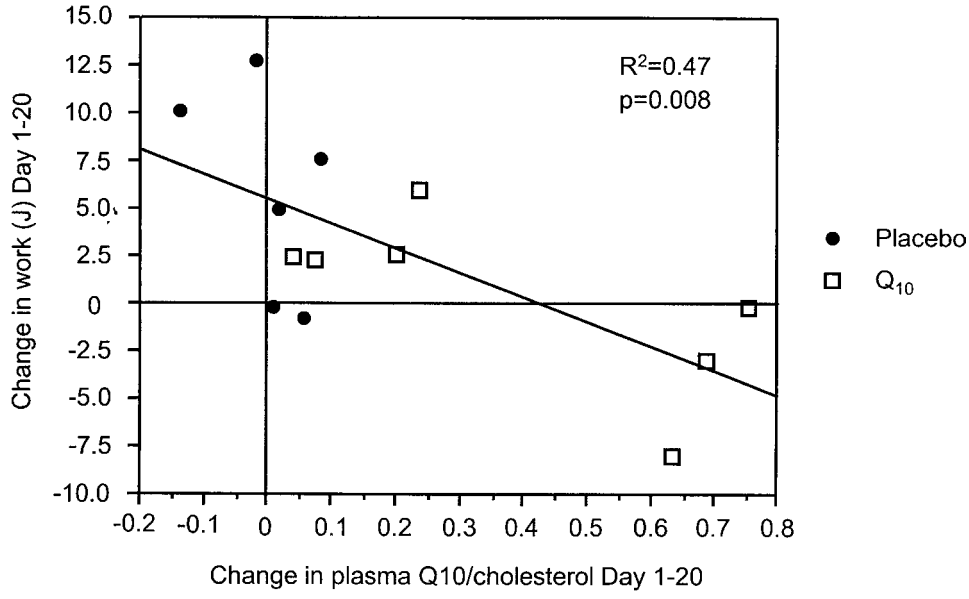

FIGURE 24.1 Relationship between changes in plasma ubiquinone-10 concentration and changes in anaerobic cycling exercise performance with 20 days of ubiquinone-10 supplementation, including 5 days of anaerobic cycling training and testing.

adaptation to anaerobic cycling training. There was no correlation between muscle Q_{10} concentration and anaerobic cycling performance at any time. One possible explanation for the negative consequences of Q_{10} supplementation in conjunction with strenuous anaerobic training could be inhibition of ROS in signal transduction.

Because only one study has been performed using anaerobic training in combination with Q_{10} supplementation, definite conclusions should not be made regarding the use of Q_{10} during strenuous physical training. Supplementation with Q_{10} for athletes that are engaged in strenuous physical exercise remains to be investigated, but with the limited data at hand, any benefits must be questioned.

24.2.3 Exercise and Heart Disease

Due to the very limited number of published studies, the effects of Q_{10} supplementation on physical performance in subjects with various heart conditions are difficult to evaluate. To our knowledge, only one study with a placebo-control group has been published.[23] In this study, exercise time and time to onset of 1 mm ST depression was significantly improved after 3 weeks of Q_{10} supplementation. The change in blood Q_{10} level was positively correlated to change in exercise duration, indicating beneficial effects of Q_{10} on physical performance of patients with stable angina pectoris. The authors of this article do, however, have some reservations regarding the Q_{10} dose, selection of subjects, and study protocol. The amount of physical training performed by the patients was also not controlled. Two studies published in edited books also report improved exercise capacity of patients with heart conditions.[17,27] In the study by Awata et al.[17] there was no placebo-control group, no statistical evaluation, and other drugs were not restricted. It also appears as if the diagnostic criteria used were not proven at the time of the study. Vanfraechem et al.[27] reportedly used a placebo-controlled study design in their investigation of physical performance and recovery in myocardial failure. Questions regarding changes in the placebo group as compared to the supplemented subjects is raised because a placebo-control group was reportedly used, but only data from the Q_{10} supplemented group presented.

In summary, data from Kamikawa et al.[23] showed some promising results in improving exercise capacity in patients with stable angina pectoris with Q_{10} supplementation. Unfortunately, limited evidence has been published so far to confirm or discard these findings.[22,25] Even though Q_{10} uptake in skeletal muscle appears limited with oral supplementation, the uptake in heart muscle tissue is unknown. Further research regarding the effects of Q_{10} supplementation in patients with heart diseases would be of interest. With at least one promising study published,[28] the possible beneficial effects of Q_{10} supplementation in subjects with various muscle diseases should also be investigated.

24.3. SUMMARY

When evaluating the effects of Q_{10} supplementation on physical exercise in humans, several aspects must be considered. Some of them are listed below.

1. Does strenuous physical exercise reduce Q_{10} levels in blood and skeletal muscle tissue, and if so, is this normal adaptation or an indication of a deficit? Decreased Q_{10} levels in blood or muscle with physical exercise have not been demonstrated in scientific studies. On the contrary, muscle and plasma Q_{10} levels in endurance trained athletes are within normal variations of ubiquinone levels in healthy individuals[15] and did not change with short-term anaerobic training.[16] In rats, ubiquinone levels in skeletal muscle tissue increased with endurance exercise.[29] As with most other substances in the body, there are optimal levels for each. Small deviation can usually be tolerated with unchanged cell function, but when levels reach above or below a set range, cell function is impaired. Before manipulating tissue levels of any substance with supplementation, the range for optimal cell function should be determined.
2. Is Q_{10} the limiting factor in any type of physical performance in healthy subjects? Based on results from published studies including physical exercise this must be questioned. Also, it is known that in situations with high H^+ concentrations, ubiquinone can function as a prooxidant.[30,31] High tissue Q_{10} concentration might therefore be sub-optimal for adaptation to strenuous physical training with significant lactate production; situations often associated with physical training at elite level training.[6]
3. Can oral Q_{10} supplementation increase the level of functional Q_{10} in human muscle tissue? Even though plasma Q_{10} levels increase with supplementation, studies have shown no increase in muscle or isolated mitochondrial Q_{10} concentration.[5,16] (See related sections in this book). Because there is evidence of positive effects of Q_{10} supplementation on physical performance of patients with heart disease, the Q_{10} levels in cardiac muscle after supplementation would be of interest to study. Perhaps the uptake differs between skeletal and cardiac muscle tissue.
4. Can the antioxidant function of Q_{10} in blood and cell membranes influence physical performance? Apparently, increase in Q_{10} concentration in blood can affect anaerobic physical performance in men.[6,16,23] In the articles by Malm et al.[6] and Svensson et al.[16] it is suggested that increased plasma Q_{10} levels interfere with signal transduction. Effects on circulating platelets with Q_{10} supplementation has been described,[32] and an interference of normal communication between muscle, endothelium, and the immune system with increased plasma Q_{10} levels cannot be excluded. In any case, it can be speculated that the proposed altered signal transduction subsequently impaired adaptation to physical training in one study,[6] and improved conditions for patients with stable angina pectoris in another study.[23]
5. How does exogenous Q_{10} influence endogenous Q_{10} production? To our knowledge, no study has investigated this question. It should be of interest because the possibility exists that endogenous production is down regulated with increased exogenous availability.

One speculation arising from the somewhat indecisive data published is that in situations where tissue or plasma levels of Q_{10} may be pathologically decreased, supplementation might be beneficial for physical performance.[23] However, increasing plasma Q_{10} concentration in healthy subjects has, at best, no positive effect on physical performance as measured in published studies.[3,5,9,13] In some situations the body's adaptation to strenuous physical training might even be diminished.[6]

It has been shown that skeletal muscle tissue Q_{10} concentration does not increase with oral Q_{10} supplementation in healthy men.[5,16] Thus, any effects of increased skeletal muscle Q_{10} concentration on physical performance in healthy subjects are unknown.

Based on current knowledge from published data, the hypotheses on positive effects of Q_{10} supplementation on physical performance in healthy men cannot be verified. Regarding the effects on women, no data are available. The laborious task of conducting studies including larger numbers of subjects (men and women) in well-designed studies remains for future research. This holds true also for other nutritional supplements, including other antioxidants.

To Q or not to Q, that is the question.

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25 Coenzyme Q and Antioxidant Potential in Athletes

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CONTENTS

25.1 INTRODUCTION

25.1.1 Significance of Antioxidant Potential in Athletes

During physical exercise large amounts of oxygen are inhaled into the body, which leads to oxidative stress.[1–10] Although the benefits of physical exercise are well established, too much aerobic exercise may even be harmful.[4,6,7] Therefore, exercising athletes have tried to improve the antioxidant potential of their bodies. They use different antioxidants including coenzyme Q to enhance the antioxidant potency to control oxygen toxicity. Athletes are also known to use vitamins and minerals to improve their athletic performance, to enhance recovery after exhaustive exercise, and to avoid respiratory infections. Other chapters in this book will concentrate on exercise induced oxidative stress and coenzyme Q and on improvement of performace by coenzyme Q. In this chapter, we focus on the antioxidant potential of athletes and the role of coenzyme Q in that potential.

Physical activity protects against atherosclerosis and coronary heart disease,[11,12] which is particularly uncommon among former endurance athletes.[13] The cardiovascular benefits of exercise are mainly explained by increased serum high-density lipoprotein (HDL) cholesterol, apolipoprotein A-I concentrations, and decreased triglyceride and LDL cholesterol concentrations.[14,15] Another possible explanation of the benefits of exercise may be decreased oxidized LDL and increased

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antioxidant potential: increasing evidence shows that endurance training may decrease the concentration of circulating minimally oxidized LDL cholesterol and may also increase LDL antioxidant potential.[16,17] Coenzyme Q carried by lipoproteins in the circulation is also suggested as playing a role in cardiovascular diseases. It has been reasoned that a low ubiquinone-to-cholesterol ratio is a risk factor for coronary artery disease and that coenzyme Q, among other antioxidants, may have an important role in the prevention of atherosclerosis.[18–21]

25.1.2 Composition of Antioxidant Potential (Role of CoQ)

The antioxidant defense system of the body has been considered to consist of several mechanisms: nonenzymatic antioxidants (such as vitamin A, C, and E, Coenzyme Q, glutathione, uric acid, albumin, bilirubin, ceruloplasmin and transferrin) and antioxidant enzymes (such as superoxide dismutases, catalase, glutathione peroxidase, glutathione reductase, and glutathione transferases).[3] Since there are so many antioxidant agents, the total antioxidant defense capacity of the tissues cannot be assessed by measuring the concentrations of only a few individual antioxidants. Therefore, methods have been developed for the estimation of the "total antioxidant capacity" and these methods are based on trapping of peroxyl radicals *in vitro*.[22–24] Although the total antioxidant capacity does not measure the antioxidant potential of the body *in vivo*, it is useful to have an estimate of the total antioxidant defense system. One problem in estimating the antioxidant potential is the distribution of the different antioxidants. For instance, less than one percent of the coenzyme Q in the body is in circulating blood, while three fourths is located in muscle tissues, heart, and skeletal muscles.[25] Therefore, when measuring the antioxidant potential from serum samples, coenzyme Q is affecting that measurement much less than in the case of muscle samples.

In serum, most of the antioxidant potential is located in aqueous phase: urate (35 to 65%), plasma proteins (10 to 50%), and ascorbic acid (0 to 24%). Only about 5 to 10 % of the antioxidant potential of the serum is lipid soluble and located in plasma lipoproteins.[26] Tocopherols are considered as the most important antioxidant in the lipid phase.[27] However, coenzyme Q has been shown to be an effective scavenger of free radicals generated chemically within liposomal membranes. When comparing the prevention of peroxidative damage, coenzyme Q was about as effective as alpha-tocopherol.[28] It was also shown that coenzyme Q can spare alpha-tocopherol when both antioxidants are present in the same liposomal membranes, but, unlike alpha-tocopherol, coenzyme Q is not regenerated by ascorbate or glutathione.[28]

25.1.3 How to Measure Antioxidant Potential

For estimation of the antioxidant capacity of human body, various indirect methodologies have been developed. Common to all these procedures, extracellular fluids (sometimes tissue extracts) are exposed to oxidizing agents, and antioxidant potential is determined by the sample's ability to scavenge oxidizing species and/or resist the oxidative challenge.[22–24,26,29] It should be kept in mind, however, that thus far, these methods have not been properly validated for the purpose, and little is known about variation of antioxidant potential even under physiological conditions.

In measurement of antioxidant potential, the most commonly used oxidative challenge is temperature-controlled decomposition of 2,2′-azobis (2-amidinopropane) hydrochloride (ABAP).[22,26] This is known to induce the production of water-soluble peroxyl radicals at a defined rate. Further, antioxidant potential is commonly indicated by the "TRAP" value of plasma (the number of moles of peroxyl radical that can be scavenged per liter of plasma). Similarly, in our studies, antioxidant potential (TRAP) is assessed by potency of serum samples to scavenge peroxyl radicals.[24] Briefly, a phosphate buffer containing 0.9% of NaCl, luminol, and the sample are mixed in a cuvette. The assay is initiated by addition of ABAP and kinetics of the reaction are followed by a chemiluminescent assay. The peroxyl radical trapping capacity is defined by the half-peak time point. Trolox (a water-soluble derivate of vitamin E; Aldrich, Milwaukee, USA) serves as a standard radical

scavenger. The coefficient of variation for the within-assay precision (20 determinations of the same serum) has been 8.1% and the coefficient of variation for between-assay precision for a period of 3 months has been 8.7%. The reference ranges using this methodology for human adults is 750 to 1340 μmol/l. Similar methodologies can be used e.g., to measure antioxidant potential from LDL cholesterol samples.

25.2 EFFECTS OF EXERCISE ON CoQ AND ANTIOXIDANT POTENTIAL

25.2.1 Effects of Acute Exercise

25.2.1.1 Serum Antioxidant Potential

Acute, even prolonged exercise does not change the concentration of serum ubiquinol-10 (Figure 25.1).[30] In trained endurance athletes, a 31-km exhaustive run did not change serum ubiquinol-10 concentration (mean $\pm$ SEM, 1.8 $\pm$ 0.2 μmol/l before the run vs. 1.8 $\pm$ 0.2 μmol/l immediately after the run).[30] Neither did serum ubiquinol-10 change during the marathon run in keep-fit marathon runners (1.2 $\pm$ 0.1 μmol/l 30 min before the run vs. 1.1 $\pm$ 0.2 μmol/l immediately after the run).[30] The ratio of reduced and oxidized ubiquinol-10 is also suggested to measure oxidative stress *in vivo*.[31] However, in the 31-km run, no changes were seen in the ratio of reduced and oxidized ubiquinol-10 (1.8 $\pm$ 0.2 vs. 1.7 $\pm$ 0.2).

Despite the lack of changes in the concentration of serum ubiquinol-10, the concentration of serum antioxidant potential increased by 22% (832 $\pm$ 29 μmol/l vs. 1018 $\pm$ 47 μmol/l) during the 31-km exhaustive run in the 8 endurance athletes, and by 16% (1165 $\pm$ 39 μmol/l vs. 1355 $\pm$ 36 μmol/l) during the marathon run in the 22 keep-fit runners.[30] The increase in serum antioxidant potential during the exercises was explained at least in part by a simultaneous rise in the concentration of serum alpha-tocopherol. Serum alpha-tocopherol rose by 29 and 7% during the 31-km run and marathon, respectively.[30] Also, both preexercise and postexercise concentrations of serum antioxidant potential and serum alpha-tocopherol correlated significantly in the keep-fit marathoners ($r = 0.47$, 95% confidence intervals 0.064 to 0.75; and $r = 0.52$, 95% confidence intervals 0.13 to 0.77, respectively).[30]

In another study, a simulated half-marathon run increased the serum antioxidant capacity and serum uric acid by 19% and 17%, respectively, in trained male runners.[32] In that study, the exercise induced increase of serum antioxidant capacity and that of serum uric acid correlated positively ($r = 0.76$, $p < 0.001$).[32] The strong exercise-induced relation between serum antioxidant capacity and serum uric acid is in line with earlier results where urate was considered as the most potent serum antioxidant in aqueous phase.[26] The concentration of preexercise antioxidant capacity also tended to correlate with maximal oxygen uptake ($r = 0.48$, $p = 0.054$).[26] This may indicate that serum antioxidant potential is elevated in response to long-term aerobic training.

In conclusion, the acute exercise induced increase in serum antioxidant potential is not influenced by changes in the concentration of serum ubiquinol.

25.2.1.2 LDL Antioxidant Potential

LDL antioxidant potential is concluded to remain unchanged during acute exercise. In the above-mentioned 31-km run and the marathon run, LDL antioxidant potential did not change (22.5 $\pm$ 1.4 μmol/mmol cholesterol before and 20.5 $\pm$ 1.3 μmol/mmol cholesterol after the 31-km run; 28.6 $\pm$ 1.9 vs. 24.7 $\pm$ 1.4 μmol/mmol cholesterol in the marathon run).[30] Neither was any change seen during acute exercises in LDL oxidation *in vivo*, as reflected by LDL baseline diene conjugation.[30] Hence, acute physical exercise, even of long duration, is concluded not to change LDL antioxidant

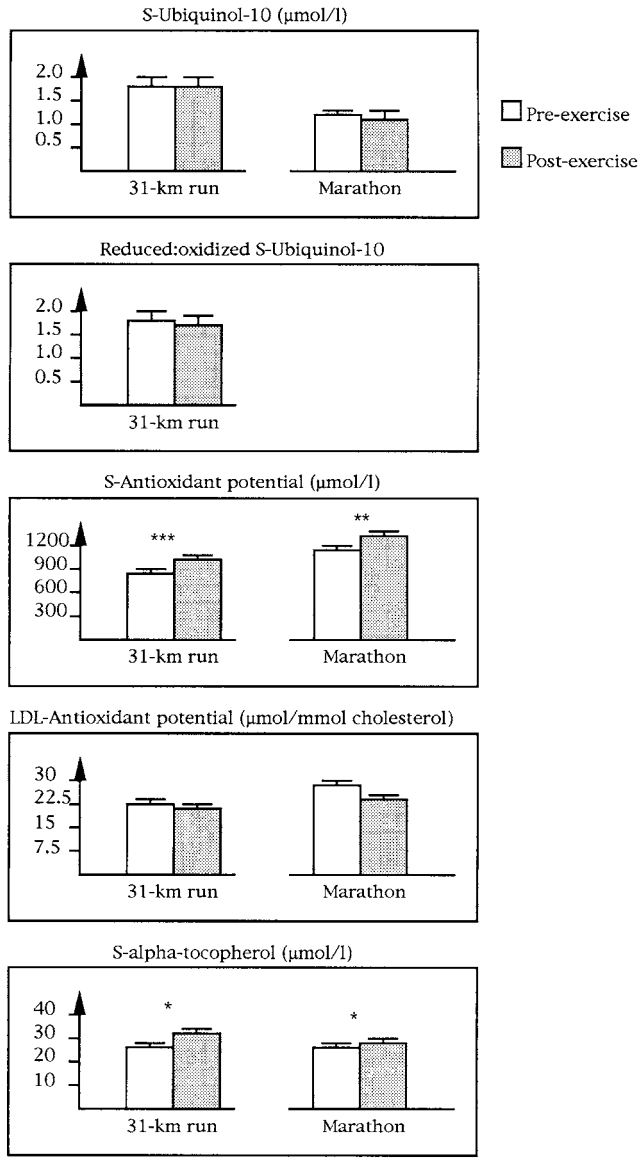

FIGURE 25.1 Concentrations of serum ubiquinol-10, alpha-tocopherol, antioxidant potential, and LDL antioxidant potential and the ratio of reduced and oxidized ubiquinol-10 before and after the 31-km run ($n = 8$) and the marathon run ($n = 22$). Mean ± SEM. Asterisks indicate statistically significant difference between samples taken before and after the runs: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

potential or LDL oxidation as measured by LDL baseline diene conjugation. Limited information is available concerning ubiquinol content of LDL cholesterol during acute physical exercise.

25.2.2 Effects of Training

25.2.2.1 Serum Antioxidant Potential

The results concerning the concentration of serum ubiquinol-10 in athletes is contradictory.[16,30,33,34] In some studies, low levels of serum ubiquinone have been measured in young endurance-trained

athletes compared with healthy sedentary subjects.[25,34–37] These low levels are suggested to be caused by a favorable lipid profile, e.g., reduced LDL cholesterol.[34] In other studies, both competing endurance athletes and veteran endurance athletes had normal levels of serum ubiquinol-10.[16,30,33] The use of anabolic androgens is shown to be associated with an increased level of serum ubiquinone levels in power athletes by an unknown mechanism.[34]

Endurance athletes or keep-fit runners with normal levels of serum ubiquinol-10 also had normal serum antioxidant potential.[30,33] Also, veteran endurance athletes had levels of serum antioxidant potential similar to their matched (age, sex, and socioeconomic status) nonsmoking, nonobese controls.[16] The concentration of serum antioxidant capacity is also suggested to correlate with maximal oxygen uptake ($r = 0.48$, $p = 0.054$).[26] In conclusion, the serum antioxidant potential of the athletes is suggested to be normal, and because of a relatively small contribution of lipid-soluble antioxidants to total serum antioxidant potential,[26] differences in serum ubiquinol level may not change serum antioxidant potential dramatically.

25.2.2.2 LDL Antioxidant Potential

Several years of intensive endurance training is associated with reduced circulating minimally oxidized LDL: the veteran athletes had 37% lower LDL fraction diene conjugation than the matched controls.[16] In that study, the veteran athletes also tended to have 17% higher LDL antioxidant potential than the controls ($p = 0.056$, NS), however, no differences were seen in concentrations of serum ubiquinol-10.[16] Other studies have also reported normal concentrations of LDL antioxidant potential in endurance athletes and keep-fit runners.[30,33] Limited information is available concerning the concentration of LDL ubiquinol-10 in athletes, although coenzyme Q is considered to be the most potent antioxidant of LDL cholesterol.[38,39]

A 10-month exercise training program for sedentary subjects increased the ratio of LDL antioxidant potential to LDL cholesterol by 16% in men and by 11% in women.[17] The decreased ratio was caused by reduced concentration of LDL cholesterol. The concentration of LDL antioxidant potential remained unchanged.[17] In that study, the concentration of circulating minimally oxidized LDL (LDL baseline diene conjugation) was also reduced by 23% in men and by 26% in women, and the decrease in LDL oxidation was also seen after adjusting the oxidized LDL by LDL cholesterol: the ratio of oxidized LDL to LDL cholesterol decreased by 14 and 18% in men and women.[17] The concentrations of serum or LDL ubiquinol-10 were not measured in that study.[17]

To conclude, in normolipidemic subjects, the LDL fraction carries about 60% of total serum ubiquinone.[34] The amount of lipoproteins may therefore be the most important factor affecting serum ubiquinone levels in humans. The effects of training, years of endurance training, and several months of exercise, on LDL antioxidant potential is suggested to decrease LDL oxidation *in vivo* and to improve antioxidant potential of LDL fraction. Therefore, physical exercise, at least endurance training, seems to act like an antioxidant producing less oxidative damage in LDL and better antioxidant potential to resist oxidative processes.

25.2.2.3 Exercise at Altitude

Altitude training has become more popular among athletes, at least in sports with a high demand for cardiorespiratory fitness. The oxidative stress and antioxidative defenses in hypoxia may be different from that at sea level, and it has been suggested that physical exercise at altitude might result in even more accelerated formation of free radicals and lead to even greater oxidative stress.[40] This hypothesis is supported by one study, where the formation of ethane and *n*-pentane by lipid peroxidation increased as the concentration of oxygen in the ambient air decreased.[41] Similarly, athletes had elevated levels of serum diene conjugation after 1 to 2 weeks stay at natural moderate altitude (1650 m above sea level) both before and after a skiing race (25 and 30%, respectively) when compared to the sea level.[42] In that study, the serum antioxidant potential tended to be lower

before the race at the 1650 m altitude compared to the sea level (1387 μmol/l vs. 1713 μmol/l, NS).[42] During the race at the 1650 m altitude the serum antioxidant potential increased by 40% (1387 μmol/l vs. 1943 μmol/l, $p = 0.016$), but there was no change at sea level (1713 μmol/l vs. 1582 μmol/l, NS).[42] The concentration of coenzyme Q was not determined.[42]

The increased serum antioxidant potential at altitude may be caused by several factors, such as changes in plasma proteins, uric acid, etc. Also, the altered energy metabolism (enhanced metabolism of glycerol and free fatty acids) at altitude may be related to the increased antioxidant potential.[43] The changed lipid metabolism in hypoxia may also have raised the concentration of lipid soluble antioxidants like alpha-tocopherol and ubiquinol-10, which are known to increase serum antioxidant potential.[30] However, further studies are needed to give any special dietary advice on possible antioxidant supplementation (like lipid soluble alpha-tocopherol and coenzyme Q) during training and racing for longer periods at moderate or high altitude.

25.3 USE OF CoQ TO ENHANCE ANTIOXIDANT POTENTIAL IN ATHLETES

25.3.1 Serum Antioxidant Potential

There is limited information available concerning supplementation of coenzyme Q in order to enhance antioxidant potential in athletes. In general, supplementation of coenzyme Q is shown to increase ubiquinol/ubiquinone content in muscles, but the results on athletes do not confirm this.[34,44–46] Also, absence of increase of coenzyme Q after supplementation is seen even in concentration of serum ubiquinol-10 in athletes.[33] The expected increase in serum ubiquinol concentration after coenzyme Q supplementation is known to depend not only on the level of serum baseline ubiquinol concentration and amount and duration of supplementation used, but also on the vehicle in which the coenzyme Q is administered. Obviously, the best way to administer the coenzyme Q is to use "plant oil capsules."

In one antioxidant cosupplementation study, endurance athletes consumed in a randomized single-blind order, either a combination of antioxidant supplements (antioxidant trial) or placebo for four weeks.[33] The antioxidant supplementation contained 294 mg of d-alpha-tocopheryl acetate, 1000 mg of ascorbic acid and 60 mg of ubidecarenon per day.[33] In that study, subjects also repeated a 31-km run twice after the four week antioxidant and placebo trial.[33] This cosupplementation markedly increased serum alpha-tocopherol concentration both at rest before the 31-km run and immediately after the run by 59 and 66%, respectively, when compared to the placebo trial (Figure 25.2) Surprisingly, no increase was seen in the concentration of serum ubiquinol-10 after the antioxidant treatment before or after the 31-km run.[33] The daily dose of coenzyme Q used might have been too low, because it has been shown that 100 mg of coenzyme Q per day may increase the amount of ubiquinol-10 about fourfold.[47] The relatively high baseline concentration of serum ubiquinol-10 may also have influenced the unchanged concentration of serum ubiquinol-10.[33] However, the preexercise and postexercise ratio of reduced to oxidized serum ubiquinol-10 decreased 26% and 38%, respectively, in the antioxidant trial compared with the placebo trial.[33]

In the antioxidant cosupplementation study, the antioxidant treatment also increased serum antioxidant potential before and after the run by 9% and 10%, respectively.[33] However, the increase in serum antioxidant potential decreased neither the preexercise serum lipid peroxidation (serum diene conjugation), nor the amount of acute exercise-induced lipid peroxidation (serum diene conjugation).[33] It was concluded that the increased serum antioxidant potential was mainly caused by a simultaneous increase in serum alpha-tocopherol concentration.[33] This is supported by an earlier study, where serum antioxidant potential correlated with concentration of serum alpha-tocopherol concentration both before and after a marathon run ($r = 0.47$ and $r = 0.52$, respectively).[30]

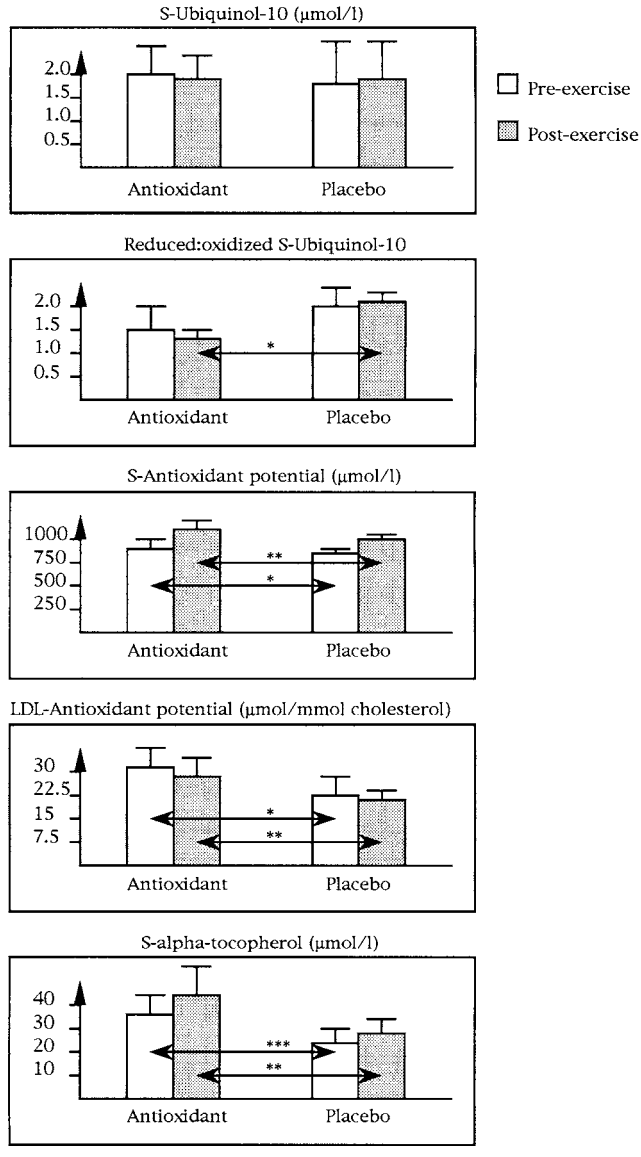

FIGURE 25.2 Concentrations of serum ubiquinol-10, alpha-tocopherol, antioxidant potential, and LDL antioxidant potential and the ratio of reduced and oxidized ubiquinol-10 before and after the 31-km run in the antioxidant and placebo trial ($n = 8$). Mean ± SD. Asterisks indicate statistically significant difference between samples taken in the antioxidant and placebo trial: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

25.3.2 LDL Antioxidant Potential

Although alpha-tocopherol is known to be the most powerful antioxidant in LDL (about one third of the total antioxidant defense of LDL),[48,49] coenzyme Q is shown to protect human LDL more efficiently against lipid peroxidation than does alpha-tocopherol.[38] Similarly, water soluble ascorbic acid dramatically increased protection against LDL oxidation *in vitro* with a physiological amount of ascorbic acid in plasma.[50] Ubiquinol is also shown to be consumed before alpha-tocopherol during LDL oxidation.[38] It is also reasonable to use supplementation of coenzyme Q when enhancing the antioxidant potential of LDL in athletes.

By using a cosupplementation of alpha-tocopherol, ascorbic acid, and coenzyme Q for four weeks (amounts mentioned above) in endurance athletes, LDL antioxidant potential increased both before and after the 31-km run by 40% and 30%, respectively, when compared to the placebo trial.[33] The marked increase in LDL antioxidant potential was suggested as being caused by the cosupplementation of antioxidants. Although a more pronounced increase in serum concentration was seen in alpha-tocopherol than in ubiquinol-10, both coenzyme Q and ascorbic acid can regenerate alpha-tocopherol.[28,50,51] Therefore, the cosupplementation of antioxidants may be more potent to enhance the antioxidant potential than the administration of a single antioxidant. Despite the significant increase in the antioxidant potential of LDL, the concentration of circulating minimally oxidized LDL *in vivo* (LDL baseline diene conjugation) did not differ between the active treatment and the placebo. Therefore, the significance of the improved antioxidant potential of LDL after antioxidant cosupplementation remains unknown. The same problem is faced in the studies measuring LDL oxidation *in vitro*: how does the reduced susceptibility of LDL to oxidation *in vitro* after antioxidant manipulation reflect the *in vivo* LDL oxidation?

25.4 CoQ TO AVOID INFECTIONS IN ATHLETES

In the last few years, our knowledge about immune systems has increased greatly. There is a hypothesis that regular training enhances immunocompetence, while intensive, exhaustive physical exercise may weaken the cellular immune system and may increase susceptibility to infections.[52] It is also proposed that a single, strenuous, and long-lasting performance increases the incidence of upper respiratory tract infections: endurance runners had a 2.1-fold increase in the incidence of upper respiratory tract infections compared to nonrunner controls in a two week period after a 56-km race.[53] Similarly, the incidence of upper respiratory tract infections in endurance runners were five-times higher during the week following a marathon run when compared to trained controls who did not participate in the race.[54]

One possible contributor to increased susceptibility to infections during exhaustive exercise may be free radicals generated during exercise. Therefore, it has been suggested that the use of antioxidants can increase the antioxidant potential and decrease the risk of infections in athletes. Earlier, ascorbic acid was shown to alleviate the symptoms and to reduce the duration of the common cold.[55–58] However, there is also some evidence that the cosupplementation of coenzyme Q and alpha-tocopherol might decrease the risk of infectious diseases in athletes.[59] In that double-blind, placebo controlled study, 32 male orienteers used both coenzyme Q and alpha-tocopherol 120 mg per day for 2 months. That study was in line with an another study, where diminished lymphocyte suppressor cell activity was seen simultaneously with decreased levels of plasma alpha-tocopherol and ubiquinol.[60]

The idea of enhanced immune system function after cosupplementation of antioxidants was not supported by another study, where young athletes used long-term (eight months) antioxidant supplementation during their normal training period.[61] Subjects in the antioxidant group took a daily oral supplement containing 1000 mg ascorbic acid, 294 mg alpha-tocopherol, and 90 mg ubiquinone, while the subjects in the control group took pills containing lactose.[61] Although no difference was seen in the incidence of upper respiratory tract infections between the groups, the results of the study were influenced by a large drop-out rate.[61] Also, the subjects of the study were mainly team sport athletes (ice-hockey and basketball) and were not engaged in very intensive endurance type training like endurance athletes.[61]

It is concluded that intensive, exhaustive physical exercise increases the incidence of upper respiratory tract infections, and that antioxidant supplementation may diminish the incidence of upper respiratory tract infections. The evidence concerning the effects of supplementation of coenzyme Q is sparse and somewhat contradictory.

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26 Exercise-Induced Oxidative Stress and Coenzyme Q

Jerzy Faff

CONTENTS

26.1 SOURCES OF REACTIVE OXYGEN SPECIES

It is very likely that both during exercise and in the postexercise period, generation of reactive oxygen species (ROS) is markedly increased. There are several potential sources of ROS that can be activated by exercise. One of the most important sources is the mitochondrial electron transfer chain. It has been calculated that 2 to 4% of the total oxygen utilized in mitochondria may undergo only one electron reduction and escape the ubiquinone step.[1] This leads to the formation of the superoxide radical (O_2), which, after additional one electron reduction produces hydrogen peroxide (H_2O_2) and may be transformed to the highly active hydroxyl radical (HO). It is well known that exercise causes a severalfold increase in oxygen utilization. In men who are moderately active physically, and whose maximal oxygen uptake (VO_2max) equals 40 to 50 ml • min^{-1} • kg^{-1} body mass and anaerobic threshold equal to 50 to 60% of VO_2max, the oxygen utilization during exercise can rise 10 to 15 times for a short period of time and 5 to 10 times for a longer time, as compared to the resting O_2 consumption (3 to 4 ml • min^{-1} • kg^{-1}). In elite, endurance trained athletes with VO_2max over 80 ml • min^{-1} • kg^{-1} and anaerobic threshold at 80 to 90% of VO_2max, the exercise oxygen utilization may be 20 to 25 times higher for a few minutes than the rest value and 15 to 20 times higher for a relatively long period. Furthermore, it should be stressed that the rise in energy metabolism from the aerobic pathway in an exercising muscle can be over a hundred times higher than the resting value. It was demonstrated that formation of the mitochondrial H_2O_2 is related to the energy production via mitochondrial electron transfer chain.[2] Therefore, it is very likely that the rise in oxygen utilization during exercise is accompanied by the elevated production of ROS.

Another potential source of ROS during exercise is probably the reaction catalyzed by xanthine oxidase within the purine degradation pathway. McCord and coworkers[3] proposed the role for this enzyme in the generation of superoxide radicals. This process involves degradation of ATP to ADP and AMP and then, in ischemic conditions, to hypoxanthine. In the reperfusion phase, hypoxanthine may be converted by xanthine oxidase to xanthine and eventually to uric acid, with the formation of superoxide radicals. There is evidence that the production of superoxide radicals via the xanthine oxidase-mediated reaction can occur both during exercise and in the postexercise period. In fact,

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a decrease in the adenine nucleotide pool and formation of inosine monophosphate in human skeletal muscles during the maximal exercise was reported.[4] The exercise-induced elevations in the plasma levels of hypoxanthine, xanthine, and uric acid were shown in many studies.[5,6] Pretreatment with allopurinol, an inhibitor of xanthine oxidase, prevented the exercise-induced elevation in the uric acid level,[6] indicating that xanthine oxidase is involved in the process. Increases in the plasma xanthine oxidase evoked by high intensity exercise were demonstrated in rats[7] and horses.[8] It has been suggested that liver is the main organ in which inosine and hypoxanthine released from the exercising muscle is metabolized to uric acid through the xanthine oxidase-catalyzed reaction.[6,9] Rádak et al.[10] reported the elevation of TBARS in the liver and kidney of rats subjected to the exhausting exercise, accompanied by an increase in the liver xanthine oxidase activity. However, Koyama et al.[6] showed that significant increases in the hepatic uric acid and TBARS contents were not detectable until 3 h after the exercise. It is possible that intensive exercise can lead to a transient ischemia due to the greatly reduced hepatic blood flow.[11,12] Consequently, during the postexercise recovery in the phase of reperfusion, conversion of xanthine dehydrogenase to xanthine oxidase may occur, activating thereby the production of uric acid.

The important source of the reactive oxygen species is mechanical or oxidative muscle damage with the initial neutrophil followed by the macrophage infiltration of the damaged tissue.[13–15] It has been established that both neutrophils and macrophages generate superoxide radicals and other reactive oxygen species and contribute to the exacerbation of the muscle damage during the postexercise period.[15–17]

Another source of ROS induced by both exercise and emotion during the pre- and postcompetition period is metabolic degradation of catecholamines.[18] In man, an increase in the plasma concentration of catecholamines has been found during both dynamic exercise of different kinds[19] and static exercise.[20]

The loosely bound iron may also constitute an important source of ROS during exercise and in the postexercise period. In the presence of the redox-potent metal ions, hydroxyl radical can be formed from the less active hydrogen peroxide and superoxide radicals via the Fenton or Haber-Weiss reactions.[21] Mechanical and free radical-induced tissue damage and destruction of erythrocytes that occurs in a variety of sports lead to the release from cells of iron-containing proteins resulting in an increased pool of relatively “free” iron. The increase in hydrogen ions may also contribute to the elevation of iron availability.[21] It has been reported[22] that exercise elevates the loosely bound iron in muscles. This elevation is associated with an increase in lipid peroxidation products. On the other hand, increased concentration of hydrogen ions promotes conversion of the weakly toxic superoxide radical into a highly active hydroperoxyl radical (HO_2).[23] It is well known that exercise, at an intensity exceeding the lactate threshold, causes the accumulation of lactate and consequently hydrogen ions. The largest increase in lactate production occurs as a result of the short-lasting supramaximal exercises.[24,25] However, even in the 1-h competitive cycle-ergometry, which relies heavily on aerobic metabolism, the blood lactate concentration may exceed 8 mM.[26]

Potential sources of exercise-related oxygen radicals also include the stimulated autooxidation of oxyhemoglobin to methemoglobin[21,27] and an increase in body temperature.[28]

26.2 EXERCISE-INDUCED OXYGEN STRESS

The effect of exercise on the generation of ROS measured with several different methods was reported in a variety of exercises. The exercise-induced increase in the lipid peroxidation indices occurred in blood and other tissues of both trained and untrained humans and animals. The methods used for demonstration of the exercise-induced oxygen stress included direct measurement of the oxygen radicals using electron paramagnetic resonance (EPR) and chemiluminescence. Other methods consisted of the estimation of the products of free radical reactions with polyunsaturated fatty acids, such as ethane and pentane as well as malondialdehyde (MDA). Total thiobarbituric acid-reactive substances (TBARS) have also been widely used as a nonspecific measure of MDA.

Davies et al.[29] demonstrated increased EPR signals in the liver and muscle homogenates obtained from animals subjected to exhaustive endurance exercise. The increased EPR signal was also seen in the stimulated contracting muscle[30] and in the rat myocardium after exhaustive exercise.[31] The exercise-stimulated enhanced generation of ROS was also detected in rat muscle using the chemiluminiscence method.[22] It was demonstrated[32] that expiratory pentane levels in humans increased after long-term submaximal exercise (50% of VO_2max). It has been established that elevation in the lipid peroxidation indices is related to the intensity and duration of the exercise and is significantly less pronounced in trained compared to untrained subjects. Kanter et al.[33] reported that both expiratory pentane and serum MDA increased as a result of the 60% VO_2max intensity work and continued to rise proportional to the increasing work load. Similar results were reported by Leaf et al.[34] who showed that in healthy, physically active men and women exposed to graded aerobic exercise, the expiratory ethane and pentane levels exceeded the lactic threshold at the resting levels, continued to rise to the VO_2max, and then declined during recovery. In this study, however, the marked elevations in expiratory ethane and pentane were not accompanied by a comparable increase in the serum TBARS levels, which were not significantly different before and after maximal exercise. According to these authors, this discrepancy suggests that MDA is cleared from the plasma by several potential mechanisms including excretion, catabolism, or redistribution to body tissues. Alesio et al.[35] demonstrated that increases in the MDA levels in skeletal muscle were more pronounced following a short-lasting exercise of high intensity than following longer exercise of lower intensity. Frankiewicz-Jóźko et al.[36] reported that in untrained rats, treadmill running until exhaustion led to elevation in TBARS in the liver, heart, and soleus muscle. In contrast, in trained animals that ran on the treadmill until exhaustion almost twice as long as the untrained animals, the rise in TBARS levels was detectable only in the liver but not in the heart or soleus. Alesio and Goldfarb[37] demonstrated that an increased MDA concentration in the muscles following moderate treadmill exercise occurred only in the untrained but not in the trained rats. In human studies, Niess[38] reported that both long distance runners as well as sedentary persons exhibit increases in their plasma MDA levels as a result of maximal exercise on a treadmill. However, the increase was substantially less pronounced in the former than in the latter group.

26.3 PHYSICAL TRAINING AND OXIDATIVE STRESS

It is well established that physical training reduces the effects of oxidative stress induced by intensive exercise and is accompanied by the changes in the antioxidant systems. It was reported that endurance training results in elevation of the muscle total superoxide dismutase (SOD), Mn-SOD, and glutathione peroxidase activities[39–41] accompanied by an increased concentration of the reduced form of glutathione in skeletal muscles[42,43] and myocardium.[44,45] Studies carried out in trained subjects showed a relationship between the total SOD activity in the vastus lateralis muscle and the maximal oxygen uptake.[46] The Cu,Zn–SOD activity in erythrocytes of well trained swimmers[47] was shown to be higher than in sedentary subjects. Notably, however, the results of studies of the effects of prolonged training on the antioxidant system vary to a great extent (for details see review by Ji and Leichtweiss,[48]).

It is noteworthy, however, that no increase in lipid peroxidation following physical exercise was reported by a number of authors. For example, Salminen and Vihko[49] could not detect any effect of prolonged running on the peroxide generation in skeletal muscles of the endurance-trained mice. Similarly, Sahlin et al.[92] described no effect of either repetitive static exercise or cycling at 60% VO_2max on the plasma MDA levels in men. Likewise, Dernbach et al.[50] did not detect any change in plasma and muscle TBARS concentrations in men and women subjected to high intensity rowing training. Drewa et al.[51] were unable to find any significant elevation in plasma TBARS in weightlifters subjected to a single bout of intensive training. Moreover, in some studies, a decrease in the plasma lipid peroxidation indices was detected after intensive exercise.[52–54] Lovlin et al.[55] demonstrated that even in untrained men, the rise in plasma MDA occurred only after exhaustive,

high-intensity exercise. In that study, exercise of lower intensity (at 40% of VO_2max) led to a decrease in the plasma MDA. In one recent investigation, no effect was reported with respect to exercise consisting of maximal cycling to exhaustion followed by concentric-eccentric contractions on the plasma MDA.[56] This discrepancy may result from the differences in training status of the tested subjects, the methods used to evaluate oxidative stress, the intensity of the exercise used, the tissues and organs tested, and/or the time that the estimation of the lipid peroxidation markers began after the exercise. However, in several other human studies, the increase in lipid peroxidation markers has been detected even at submaximal work load[32,22,33,34,57,58] and was demonstrated even in well-trained subjects.[38,58,63]

It was shown that exercise-induced oxygen radical formation adversely affects the nuclear DNA prepared from lymphocytes.[64] Likewise, increased urinary excretion of 8-hydroxy deoxyguanosine, a marker of DNA damage, was described as a result of a marathon race.[63] However, other authors could not detect any changes in the urinary 8-hydroxy deoxyguanosine excretion over 3 days after the 20-km run or after the moderate-intensity cycling performed over three consecutive days.[66,67]

26.4 EFFECTS OF CoQ_{10} ON EXERCISE-INDUCED OXIDATIVE STRESS

It was repeatedly indicated that application of CoQ_{10} led to a marked elevation of both the oxidized and reduced forms of ubiquinone in various animal tissues[67,68] and in human plasma[69] resulting in protection against lipid peroxidation induced by factors other than exercise.[68,71,72] It was demonstrated that CoQ_{10} is about as effective in preventing oxidative damage to lipids as α-tocopherol.[72] According to other authors, CoQ_{10} is a more efficient antioxidant than vitamin E.[74] Coenzyme Q_{10} has been demonstrated to spare α-tocopherol when the two antioxidants were present in the same liposomal membrane,[72] as well as to recycle vitamin E.[74] In addition, it was shown[75] that pretreatment with CoQ_{10} inhibited the release of inosine and hypoxantine from rabbit hearts subjected to hypoxic perfusion followed by reoxygenation, and decreased the inosine and adenosine contents in the myocardium. These effects of CoQ_{10} during hypoxia and reoxygenation could account for the attenuation by CoQ_{10} of the exercise-induced generation of superoxides in the course of the hypoxantine metabolism.

In view of the above-cited evidence of the excellent antioxidant properties of CoQ_{10}, the beneficial effect of this drug on the exercise-induced increase in the lipid peroxidation markers should have been expected. It was shown[76] that supplementation of rats with CoQ_{10} (10 mg•kg^{-1} body mass for 4 weeks) prevented or significantly suppressed the exhausting exercise-induced increase in TBARS in the liver, heart, and red and white portions of the gastrocnemius muscle (Figure 26.1). Additionally, treatment with CoQ_{10} prevented the exercise-induced decrease in the reduced glutathione in the rats' livers and hearts, as judged by the levels of the nonprotein sulfhydryl groups (Figure 26.2). In the trained cyclists, however, Braun et al.[77] detected no effects of ubiquinone on the serum concentration of malondialdehyde. In the study by Laaksonen et al.[78] neither the ubiquinone supplementation nor the exercise affected the serum malondialdehyde concentration in either the young or older endurance-trained athletes. Kaikkonen et al.[69] tested the effects of the combined application of coenzyme Q_{10} (90 mg daily) and *d*-α-tocopheryl acetate (13.5 mg daily for 3 weeks) in the moderately trained marathon runners. They showed that prior to the run, the combined treatment led to elevation in the plasma CoQ_{10} concentration by 282% accompanied by the significant reduction of susceptibility of the plasma VLDL + LDL fraction to copper-induced oxidation. However, the supplementation had no effect on lipid peroxidation caused by the marathon run as assessed by the increase in negatively charged LDL and the level of copper-induced oxidation of VLDL + LDL.

Effects of CoQ_{10} on the additional selected phenomena that could be, at least partially, related to the exercise-induced oxygen stress were also studied. In fact, several authors[79, 88] reported enhancement by the drug of the work capacity in patients with coronary heart disease, probably as a result of the effect of the drug on the ATP metabolism.[76] However, supplementation with CoQ_{10} could prevent or minimize tissue damage caused by the oxidative stress and thereby influence

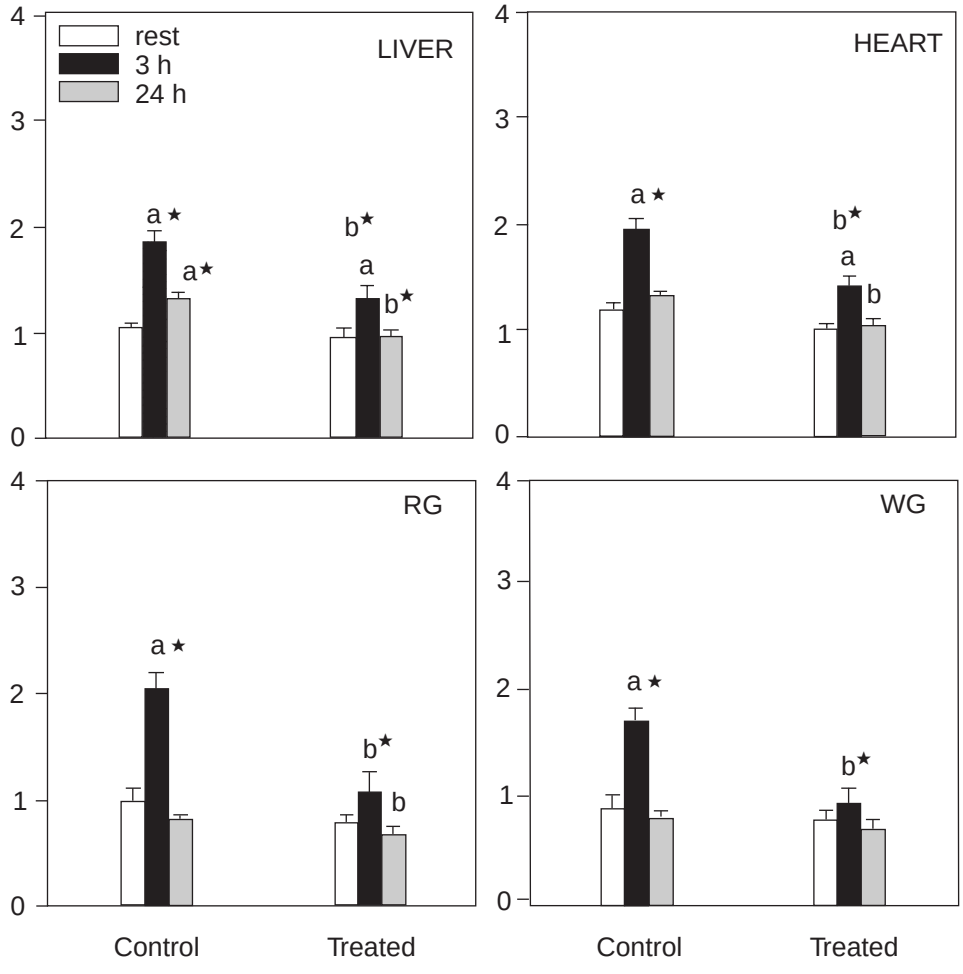

FIGURE 26.1 Amounts of thiobarbituric acid-reactive substances in the liver, heart, and white (WG) and red (RG) part of the gastrocnemius muscle at rest, and at 3 and 24 h after exercise in control and CoQ_{10}-treated rats. Values are means and SEM ($n = 10$ per group); a and *a are significant differences between the resting and the exercise values at $P < 0.05$ and $P < 0.01$, respectively; b and *b are significant differences between the control and the treated groups at $P < 0.05$ and $P < 0.01$, respectively. From Faff, J., and Frankiewicz-Jóźko, A., *Eur. J. Appl. Physiol.*, 75, 413, 1997.

physical performance of the patients. This effect of CoQ_{10} on the performance in athletes repeatedly subjected for years to oxidative stress during exhausting training sessions and competitions should also be expected. The first studies of the CoQ_{10} supplementation gave excellent results. Amadio et al.[87] administered CoQ_{10} to basketball players for 40 days and reported the VO_2max increased by as much as 12 ml • min^{-1} • kg^{-1}. Beneficial effects of the CoQ_{10} treatment on physical performance and/or VO_2max were also described by Guerra et al.,[82] Van Fraechem and Folkers,[83] and Zeppilli et al.[80] However, a number of other studies carried out in healthy trained and untrained men did not reveal any significant effects of the ubiquinone treatment on VO_2max,[77, 84–86] lactate threshold,[87] or total work done during the exercise test.[77,78,84,87,88] Moreover, no effects could be detected of the combined CoQ_{10}, vitamin C, and vitamin E supplementation on energy metabolism in muscles or muscle fatigability in triathletes.[86] In our studies,[84] administration of CoQ_{10} for four weeks to young, healthy men subjected to intensive work for ten weeks slightly improved their capacity to perform a short, supramaximal exercise. In contrast, as reported by Malm et al.[85] the increase in anaerobic

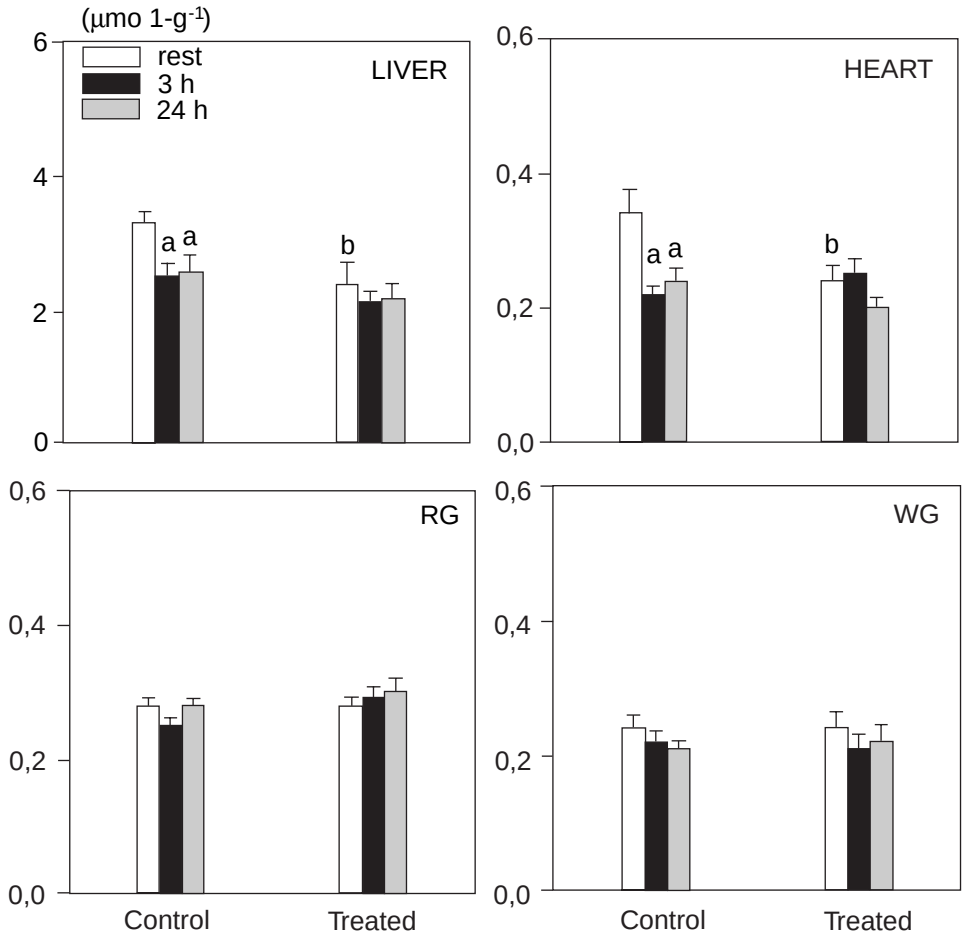

FIGURE 26.2 Amounts of nonprotein sulfhydryl groups in the liver, heart, and white (WG) and red (RG) parts of the gastrocnemius muscle in control and CoQ_{10}-treated rats at rest, and at 3 and 24 h after exercise. Values are means and SEM ($n = 10$ per group), a is a significant difference between the resting and the exercise values ($P < 0.05$); b is a significant difference between the control and the treated groups ($P < 0.05$). From Faff, J., and Frankiewicz-Jóźko, A., *Eur. J. Appl. Physiol.*, 75, 413, 1997.

physical performance caused by anaerobic training was less pronounced in the CoQ_{10}-supplemented than in the placebo-treated subjects.

Many factors associated with physical exercise have been postulated to stimulate the release of cellular enzymes into the blood stream. These factors include mechanical tissue damage, decrease in the cellular energetic compounds,[89] acidosis,[91] influx of lymph, and presence of soluble muscle proteins in the interstitium.[91] In addition, it was suggested that the postexercise elevation of cellular enzymes in the plasma is related to the increased membrane permeability caused by lipid peroxidation.[29] Indeed, a correlation was described between the release of muscle enzymes, neutrophil infiltration of muscles, and enhanced production of superoxide radicals by neutrophils *in vitro*.[92] In the electron spin resonance studies of rat skeletal muscles, Jackson et al.[30] showed that after extensive contraction of the muscle, the creatine kinase efflux correlated with the enhanced free radical signal. The correlation between the plasma TBARS and the serum creatine kinase levels was detected in runners after an 80-km race,[59] in speed skaters exercised on a cycloergometer,[53] and in weightlifters after training sessions.[51] A relationship was also reported between the TBARS erythrocyte concentration and the serum creatine kinase levels in the weightlifters tested by Drewa

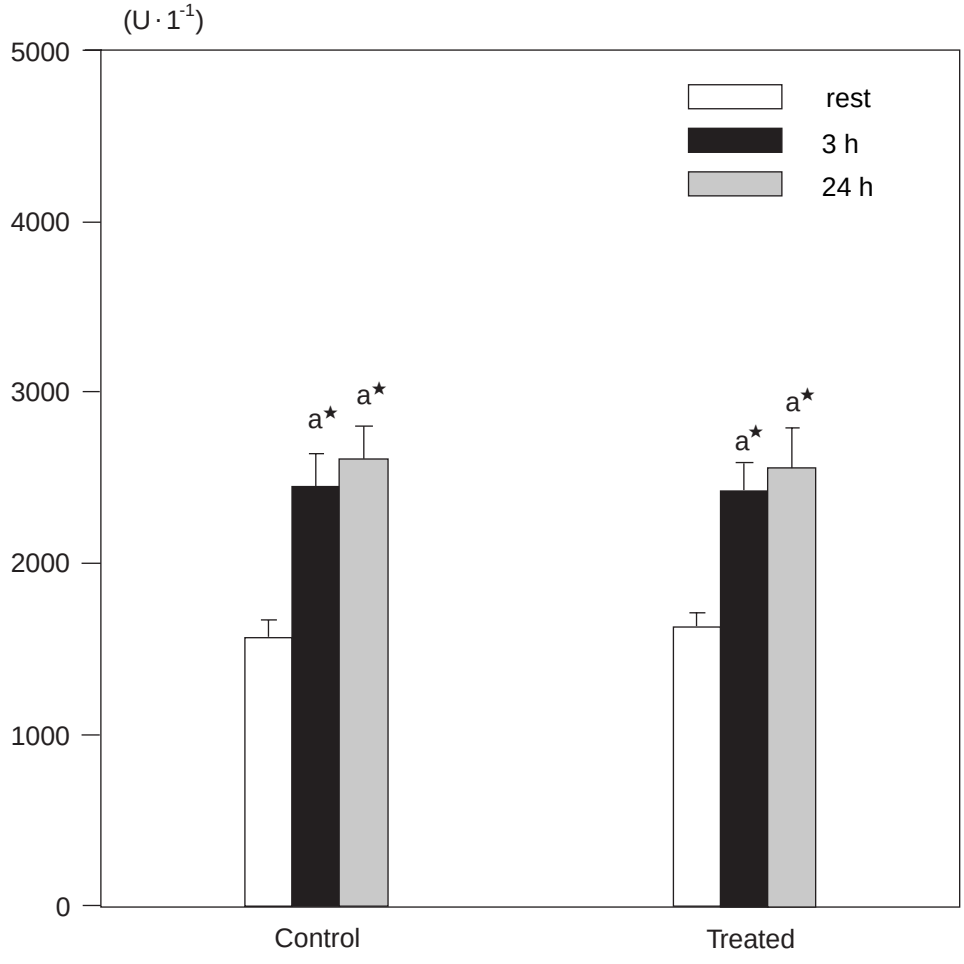

FIGURE 26.3 Serum concentration of creatine kinase at rest, and at 3 and 24 h after exercise in the control and CoQ_{10}-treated rats. Values are means and SEM ($n = 10$ per group) *a is a significant difference between the resting and the exercise values ($P < 0.01$). From Faff, J. and Frankiewicz-Jóźko, A., *Eur. J. Appl. Physiol.*, 75, 413, 1997.

et al.[51] Takeo et al.[75] reported that pretreatment with CoQ_{10} completely inhibited the release of creatine kinase from rabbit hearts exposed to hypoxia and reoxygenation. Shimomura et al.[68] showed that CoQ_{10} administered to rats suppressed the elevation of plasma creatine kinase and lactate dehydrogenase caused by downhill treadmill running. The effect of treatment, however, was observable only immediately after the exercise and not at the 40th hour after the exercise. According to the authors, these results suggest that the CoQ_{10} treatment protected skeletal muscles against injury caused by exercise but not against damage inflicted by the exercise-induced inflammatory processes. In contrast, no apparent effect of CoQ_{10} treatment on the increased serum creatine kinase activity in rats running uphill could be seen.[76] In human studies, the CoQ_{10} supplementation to soldiers for four weeks did not affect the elevated serum activities of creatine kinase and aspartate aminotransferase resulting from intense military training.[84] Likewise, Kaikkonen et al.[69] could not detect any effect of the combined CoQ_{10} and vitamin E supplementation on the exercise-induced increase of creatine kinase in the serum. Finally, Malm et al.[85] showed that in men subjected to strenuous anaerobic exercise, application of CoQ_{10} led to a higher elevation of the plasma creatine kinase as compared to the placebo-treated counterparts.

The influence of supplementation of elite Swedish athletes with CoQ_{10} and vitamins E and F was extensively studied by Karlsson.[93] It has been found that antioxidant therapy improved physical health of the athletes as assessed by the decreased number of days missed from training due to disease. It is very likely that this effect of antioxidant therapy in athletes repeatedly subjected to exhausting training sessions and competitions has been related to the prolonged protection against reactive oxygen species.

26.5 CONCLUSIONS

It is well established that physical exercise leads to an increased generation of reactive oxygen species and consequently to lipid peroxidation. Elevation in lipid peroxidation indices is related to exercise intensity and duration and is significantly less pronounced in trained subjects than in untrained ones. A number of studies have shown no effect of physical exercise on the lipid peroxidation parameters. Other authors, however, described exercise-induced increases in the lipid peroxidation indices even in well-trained athletes after submaximal exercises. Pretreatment of rats with CoQ_{10} markedly suppressed the exercise-induced increase in the markers of lipid peroxidation in the heart, liver, and gastrocnemius muscle. In contrast, a number of other studies carried out in healthy, trained and untrained men did not reveal any significant effects of ubiquinone treatment on lipid peroxidation markers induced by exercise. It is possible that physiological tissue levels of ubiquinone in healthy men can effectively resist exercise-induced oxidative stress. On the other hand, it is possible that ubiquinone treatment prevents oxidative tissue damage even though this effect may be undetectable when assessed in men in terms of changes in lipid peroxidation parameters in the blood. Physical training markedly reduces lipid peroxidation induced by intensive exercise. However, prolonged heavy exercise increased the consumption of certain antioxidants including CoQ_{10}. Therefore, in athletes and other physically hard-working men, the supplementation with CoQ_{10} combined with other antioxidants, seems to be desirable.

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Section 3E

Aging

27 Coenzyme Q Supplementation and Longevity

Hannu Alho and Kimmo Lönnrot

CONTENTS

27.1 INTRODUCTION

During the last four decades there has been much debate on the role of free radicals in aging. According to the theory of free radicals in aging, first introduced by Harman[1] and Gerchman et al.,[2] all biological systems involve oxidative stress originating as a result of an imbalance between the generation of oxidizing species and cellular antioxidant defense. This can cause damage by peroxidation to all cellular macromolecules, including proteins,[3] DNA,[4] and lipids,[5] thus leading to the cellular degeneration and damage related to aging. This theory has led to the suggestion that antioxidants such as ubiquinol may play a role in the prevention of the aging process.

Decline of energy metabolism can be considered as one mark of aging in many different types of cells, especially liver and muscle cells. Weinbach and Garbus[6] were the first ones to suggest the decrease in the efficiency of oxidative phosphorylation by reporting decreased 3-hydroxybutyrate oxidation in liver mitochondria from aged rats. Since then, concentrations of many high-energy components, for example ATP/ADP ratio[7,8] and creatine-phosphate content[9] in the heart and muscle of rats, have been reported to decrease with increasing age in mammals. Activities of some rate-limiting

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TABLE 27.1
The Direction of the Change in Coenzyme Q Concentrations in Different Rat Tissues during Aging

	Heart	Liver	Spleen	Kidney	Brain	Lung	Muscle
Beyer[a]	↓	↑	–	↑	↓	n.s.	↑
Kalen[a]	↑	↓	↓	↓	–	↓	–
Lenaz[a]	n.s.	n.s.	–	↑	–	–	n.s.

[a] Beyer et al. (1985) measured the total coenzyme Q concentrations, while Kalen et al. (1989) and Lenaz et al. (1993) measured coenzyme Q_9 concentrations. The results from Lenaz et al. are from mitochondrial fraction, while the results from Beyer et al. and Kalen et al. are from tissue homogenate.

enzymes in the ATP synthesis, for example hexokinase and phosphofructokinase,[7,10] have also been reported to decrease with age in rat tissues, although no age-associated changes in these enzyme activities in humans and rats have been reported.[11] Interestingly, studies of mitochondrial ultrastructure in the heart and liver tissues of mice and humans have indicated that during aging the number of mitochondria per unit of cell area[12–14] as well as the sectional area of individual mitochondria decrease[15] while the number of giant mitochondria increases,[12] further suggesting a decline of the function of mitochondria with increasing age.

27.1.1 Coenzyme Q and Longevity

It has been proposed that the tissue content of ubiquinone also decreases during aging,[16,17] which may in part be responsible for the decline of energy metabolism. As previously pointed out, many obligatory components of mitochondrial respiratory chain decrease with age. Changes brought about by increasing age in coenzyme Q tissue concentration have been reported in both human and rat tissues by three different authors.[17–19] The direction of changes in different rat tissues is summarized in Table 27.1. Kalen et al. studied the rats only until 300 days of age, although the average lifespan of a laboratory rat is about 24 months (730 days). Also the HPLC (high performance liquid chromatography) methods used in these studies were different, which makes it difficult to compare the numeric results between the three studies.

The only attempt to study the issue of chronic coenzyme Q treatment on survival has been made by Bliznakov.[20] In his study, 16 to 18 month-old mice were given weekly intraperitoneal administration of 50 μg of coenzyme Q_{10}. The mean survival time of mice was increased from 20 months in the control group to 31.2 months in the treatment group. However, in this study there were no other parameters reported including coenzyme Q_{10} concentrations in plasma and different tissues. Also, this study has not been repeated subsequently.

These observations have led to a presumption that dietary supplementation might be beneficial with aging. Even an increased survival has been suggested to result from coenzyme Q injections in mice.[20] Furthermore, many short-term experimental animal studies indicate that exogenous coenzyme Q treatment may be beneficial in some cardiovascular diseases, such as recovery of myocardium and endothelium from postischemic reperfusion injury,[21–23] a phenomenon common to myocardial infarction. In these studies, however, the administration of coenzyme Q has most commonly been parenteral, which is not as physiological as oral administration. Prior to our study, there was no previous data on the effect of long-term coenzyme Q administration and no reports about its effects on development and survival. We recently studied and published the effect of oral supplementation of ubiquinone Q_{10} on the survival of both rats and mice.[24,25] We followed both populations from birth to death and monitored their growth[24] and survival. In order to ensure that our observations were relevant and due to ubiquinone, we also measured ubiquinone Q_9 and Q_{10} concentrations in plasma and different tissues.

27.2 MATERIALS AND METHODS

27.2.1 Lifelong Supplementation of Coenzyme Q_{10}

In order to identify differences in survival and longevity, we followed all 150 male Sprague-Dawley rats and 86 male c57/B17 strain mice throughout their life spans. Both rats and mice were randomly assigned to a study group receiving 10 mg/kg/day of coenzyme Q_{10} or control group receiving a standard diet.

Ubiquinone Q_{10} was mixed into the normal animal diet by using soybean oil as a vehicle. Soybean oil was also added to the control food. All the food was kindly provided by Pharma Nord (Vejle, Denmark). The feeding was adjusted so that the daily intake of Q_{10} was 10 mg/kg/day in the experimental group and less than 0.5 mg/kg/day in the control group. For rats weighing less than 150 g, the quantity of food made available was 20 g/rat/day; for rats weighing over 150 g, the amount was 25 g/rat/day. For the mice, the amount was 5 g/mouse/day.

Animals were regularly weighed and inspected to follow their growth and general well-being. Also, survival of animals was followed. An autopsy was performed on all rats that died naturally, whenever possible within 24 hours. A total of 31 treated and 29 control rats were autopsied. An autopsy included a macroscopic evaluation of skin, internal tumors, and pathology. After macroscopic evaluation, samples were taken of heart, liver, kidney, lung, hypophysis, adrenals, and tumors for later microscopic examination. This included a normal pathological examination of tissues as carried out by a pathologist.

27.2.2 Coenzyme Q Measurements in Plasma and Tissues

Coenzyme Q concentrations in plasma, CSF, and different tissues were measured by the high performance liquid chromatography (HPLC) method as described by Lang et al.[26] The serum samples were extracted with *n*-propanol and coenzyme Q_7 was added as an internal standard. The coenzymes were reduced with $NaBH_4$ prior to HPLC employing a Gilson 232-401 automated sampler (Gilson Medical Electronics Inc., Villiers le Bel, France). The HPLC equipment consisted of two Wallac 2258 pumps (Pharmacia Biotechnology, Uppsala, Sweden), a Beckman Gold C18-ultrasphere column (Beckman Instruments Inc., CA, USA), a Gilson C18 precolumn, and an ESA electrochemical detector (ESA Inc., MA, USA). Coenzyme Q concentrations in the heart tissue were measured according to the method described by Lang et al.[26] with some modifications. An identical piece of left ventricle from each heart was dry homogenized with a microdismembrator (Micro-Dismembrator, B. Braun, Melsungen, Gemany). An accurate amount of ventricle was dissolved in 300 μl of 1:2 ethanol-water solution. We added 100 μl of methyl substituted Q_{10} (0.5 g/l, dissolved in ethanol) as an internal standard. The tissue samples were stirred with an ultrasonicator, and coenzyme Q was extracted into 500 μl of hexane, which was dried under nitrogen and resuspended into 200 μl of methanol-ethanol (80:20). UV detection of reduced and oxidized coenzyme Q was performed by high performance liquid chromatography (HPLC) under the following conditions; pump: LKB 2249, column: Chromsphere C-18, mobile phase, methanol-ethanol 80:20, flow rate: 0.5 ml/min., Detector: LKB 2141, wavelength: 275 nm. The standard samples of Q_9 and Q_{10} were extracted and analyzed accordingly as the heart tissue samples.

27.2.3 Microscopic Examinations

A fluorescence histochemistry of adrenal gland and superior cervical ganglia was carried out to estimate lipopigment accumulation. This was determined by a quantitative fluorescence microscopy as described in detail elsewhere.[27] Briefly, the fixed tissues were embedded in paraffin, sectioned serially, and examined under a Nikon Mikrophot FXA fluorescence microscope. Quantitation of pigment autofluorescence was performed with an image analyzer (DPS-200 MTI image processor with Microscale software). Autofluorescence intensity was measured at random from 80 sympathetic

neuronal pericarya or cells of adrenal cortex at four different levels. The data are expressed as mean arbitrary units.

After an autopsy, samples of heart, liver, kidney, lung, hypophysis, adrenals, and tumors were taken for later microscopic examination. Samples were immediately immersion fixed (4% paraformadehyde for 2 to 6 h at room temperature) and stored at +4°C. Later on samples were embedded in Tissue-Tek (Miles Inc., Elkhart, USA) and sectioned into 10 μm slices with a cryotomy (Micron, Heiselberg, Germany). After staining the slices with hematoxylin-eosin, a standard pathological examination was carried out by a pathologist.

27.3 RESULTS

27.3.1 Survival and Morbidity of Rats and Mice after Lifelong Supplementation

Supplementation showed no teratogenic effects in rats. There were no differences in weight gain or growth in either rats (Figure 27.1) or mice. For rats, the percentage cumulative mortality did not significantly differ between the groups (Figure 27.2), and the average survival in the control group was 26.5 months and in the supplemented group 24.3 months (Figure 27.3A). For mice the average survival times in control and supplemented groups were 28.1 and 29 months, respectively (Figure 27.3B). Survival analysis indicated a tendency toward longer survival in the control rats ($P = 0.073$) when compared to supplemented rats. In mice there were no differences in survival between groups.

The Q_{10} concentrations were 2.6 to 8.4 times higher in the plasma (p value ranging from 0.0001 at 6 month to 0.0269 at 18 month) and 3.2 to 6.6 times higher in the liver (p value ranging from 0.0002 at 6 month to 0.0619 at 18 month) at all ages in the Q_{10} supplemented group than in the control group (Figure 27.4). At 18 months in liver, the Q_{10} concentration was not statistically different ($p = 0.0619$) because of a wide standard deviation and a small sample number. Interestingly, the plasma and liver Q_9 concentrations were also higher in the Q_{10} supplemented group at ages 18 and 24 months than in the control group. In plasma, Q_9 concentration was 1.9 times higher at 24 months in the treated group than in the control group ($p = 0.0013$). In liver, the Q_9 concentration was 1.7 times higher at 18 months ($p = 0.036$) in the treated group and 1.8 times higher at 24 months ($p = 0.037$) than in the control group. In kidney, heart, and brain tissues, there were no differences between the control and the treatment groups in either Q_9 or Q_{10} concentrations, except that at 18 months, the Q_{10} concentration in kidney was higher in the experimental group than in the control group.

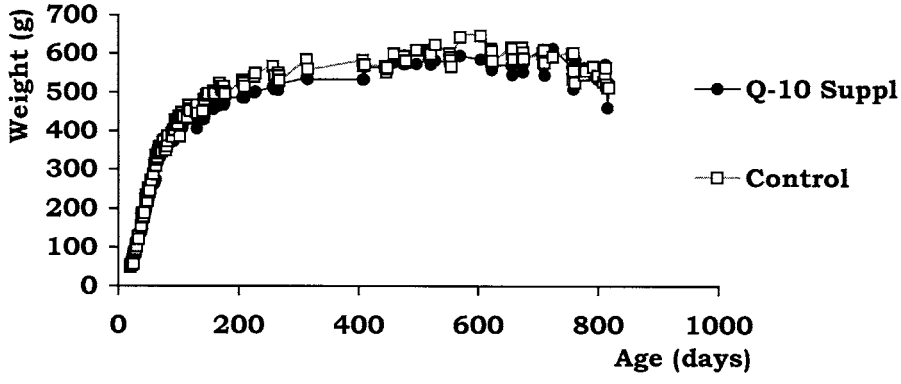

FIGURE 27.1 The monthly measured gain in body weight. Each point represents the mean of body weight of all animals in each group. Modified from Lönnrot et al., 1995.

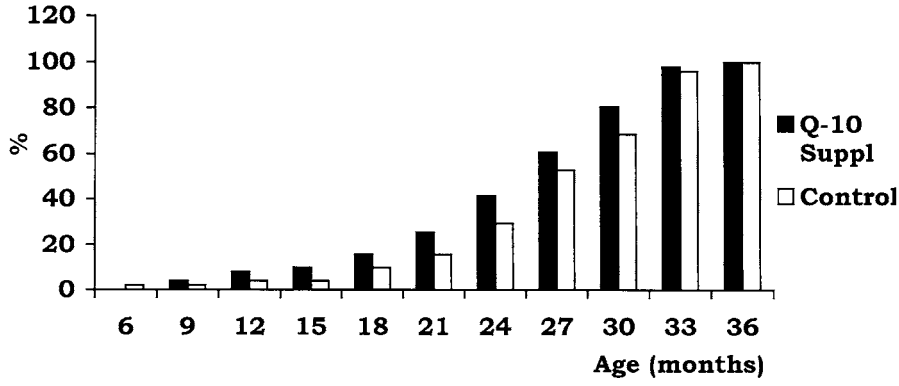

FIGURE 27.2 The percentage cumulative mortality at the end of the experiment. From Lönnrot et al. 1995.

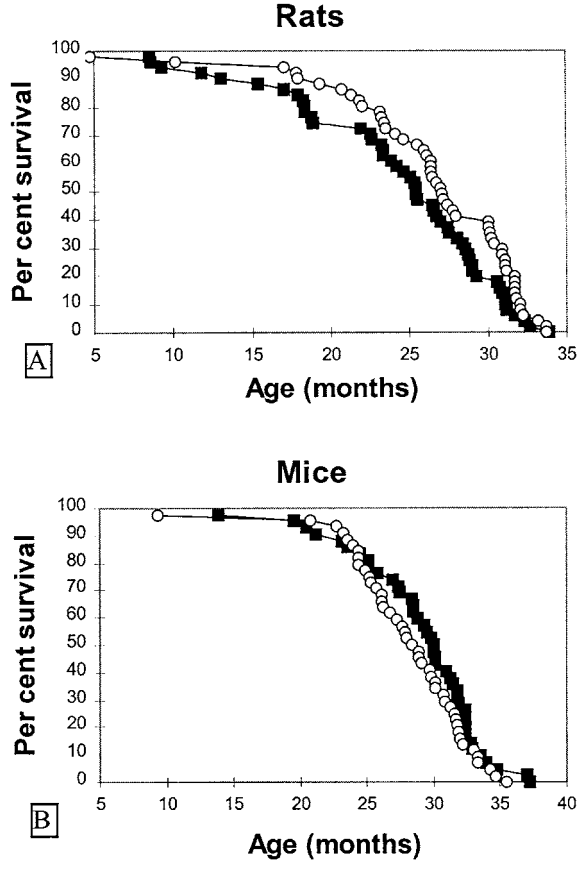

FIGURE 27.3 Percentage survival of Q_{10} supplemented ■ and control ○ rats (A) and mice (B). Mean for experimental rats: 24.3 *SD* ± 0.91 months; and for control rats 26.5 ± 0.83 months; $p = 0.073$. Mean for experimental mice: 28.1 ± 0.70; and control mice 29.0 ± 0.74 months; $p = 0.24$. From Lönnrot et al., 1998.

In the histological examination of the rat tissues, alveolar histocytosis was observed in 31% of supplemented rat lungs and only in 9.7% of control lungs. However, the difference was not statistically significant (incidence proportion ratio, IPR 3.2, 95% confidence interval, CI 0.96 to 11). Also, macroscopic evaluation revealed 39% of supplemented rat kidneys to contain renal

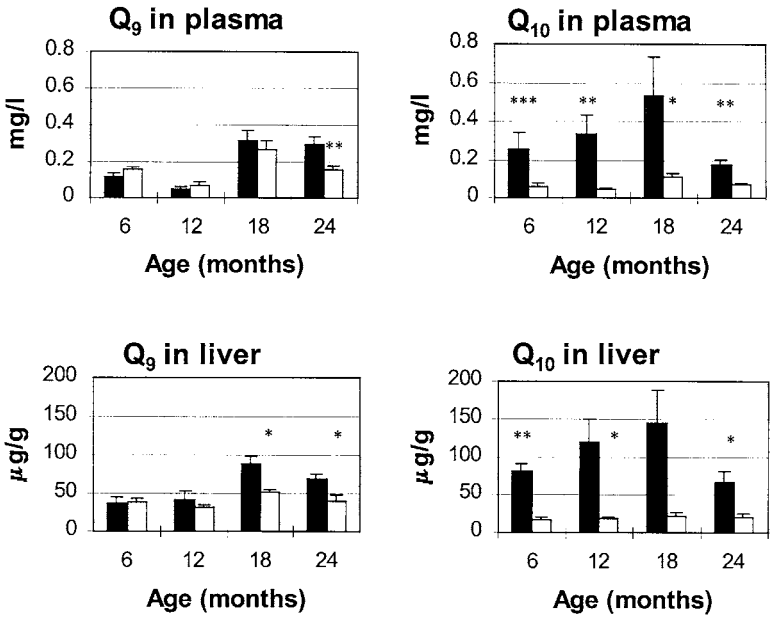

FIGURE 27.4 Total ubiquinone Q_9 and Q_{10} concentrations in rat plasma and liver at 6, 12, 18, and 24 months. In plasma, $n = 10$, 4, 4, and 4 animals per group, respectively. In liver, $n = 4$ animals per group. □ = control, ■ = Q_{10} treated. Values are μg/g wt weight ± SD in liver and μg/l ± SD in plasma. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to adjacent control group. From Lönnrot et al., 1998.

stones while only 21% of control kidneys had stones. This difference was not statistically significant (IPR 1.8, CI 0.78 to 4.2). Furthermore, renal stones were not connected to increased renal cystic changes or interstitial nephritis. There were no differences between groups in other tissues examined.

Lipopigment accumulation was examined in rats at the age of 12 and 18 months in adrenal cortex and superior cervical ganglia by calculating the number of pigment grains and by measuring the relative autofluorescence intensity by an image analyzer. A normal accumulation of lipopigment in superior cervical ganglia and of pigment in the cortex of the adrenal gland was observed in both groups. Supplementation had no effect on the number or on the intensity of the pigment grains.

27.4 DISCUSSION

27.4.1 Effect of Lifelong Supplementation on Survival and Morbidity of Rats and Mice

Our longevity study is the first attempt to measure the effect of ubiquinone supplementation on the whole life span and survival of rats or mice. Bliznakov et al. reported a significant increase in the life span of mice after weekly coenzyme Q_{10} injection.[20] However, these results have not been confirmed since. Previously Bliznakov et al. had observed decreased activity of succinate dehydrogenase-coenzyme Q complex in these mice.[28] Thus it is possible that these mice had a coenzyme Q deficiency and the replacement of the deficiency resulted in an increase in survival. In our study, lifelong supplementation with coenzyme Q_{10} did not result in an increase in survival of either rats or mice. In addition, we did not observe any significant differences in the macroscopic or histopathological examination of rat tissues. Most of the coenzyme Q content in rats and mice is Q_9. It

is unlikely, although possible, that the use of Q_9 instead of Q_{10} would have yielded a different result. Kagan et al.[29] have suggested that coenzyme Q homologues with long isoprenoid side-chains (Q_6–Q_{10}) are much less efficient in preventing membranes from lipid peroxidation than the short-chain homologues (Q_0–Q_5). However, the antioxidant efficiency between different long-chain homologues is only slightly different.[29] On the other hand, Fiorentini et al.[30] reported that Q_3 and Q_7 exhibited a similar antioxidant function in their study and suggested that the lack of incorporation of long-chain coenzyme Q homologues into biomembranes by the method used in the study by Kagan et al.[29] might have been the reason for contradictory results. In our study, Q_{10} was chosen, since it could be considered to represent exogenous coenzyme Q incorporation into tissues. In addition, the total amount of coenzyme Q was rather large and the availability of Q_{10} was better than Q_9.

27.4.2 Effect of Age and Supplementation on Plasma and Tissue Coenzyme Q Content

Three previous reports[17-19] of tissue and plasma coenzyme Q concentrations during the aging of rats have given contradictory results. Our results also vary from the previous studies. All of these studies have utilized different methods. Beyer et al.[18] used a dual-wavelength recording photometer and measured absorbance differences between oxidized and reduced samples.[31] Lenaz et al.[19] do not specify in their report what method they have used. Kalen et al.[17] used HPLC with ultraviolet light detection. In our studies, we used HPLC with electrochemical detection. The most accurate method of previous studies, and closest to our method, is in the work by Kalen et al. They reported an increase in Q_9 concentration in the heart and a decrease in Q_9 liver and kidney concentration. None of these were observed in our study. We observed an increase up to 12 months in the heart's Q_{10} concentration, but not Q_9, and an increase up to 18 months in liver's Q_9 and Q_{10} concentration followed by a decrease. Unfortunately, Kalen et al. did not measure Q_{10} concentrations in their study. Also, they studied rats only up to 300 days of age while the mean survival of a laboratory rat is about 24 months (730 days). Therefore one cannot draw further conclusions by comparing these studies.

Supplementation in humans, rats, and mice was well tolerated and no side effects were reported or observed. In all of our studies there was a significant increase in the plasma concentration of Q_{10} in the supplemented group. In agreement with the previous studies[32–35] we observed a significant increase in the liver Q_{10} concentration in rats after supplementation. Zhang et al.[34] reported that Q_{10} taken up by liver is mainly located in the lysosomal compartment. We did not measure subcellular fractions and thus could not confirm this result. Interestingly, in our study coenzyme Q_9 liver concentration was also increased in the supplemented rats at the ages of 18 and 24 months. A similar increase of liver Q_9 concentration has been reported by Lenaz et al.[19] after perfusion of rat liver with Q_{10}-containing perfusate. Since coenzyme Q_9 is the natural quinone in rats, exogenous Q_{10} is thought to represent a label of exogenous coenzyme Q incorporation. Lenaz et al. suggest that the higher level of Q_9 is a result of protection of endogenous coenzyme Q from oxidative damage by extramitochondrial Q_{10}. However, this hypothesis awaits further confirmation.

Lifelong Q_{10} supplementation did not result in an increase in Q_9 or Q_{10} concentration in the heart in our studies. This is in agreement with several other studies,[33–37] although there are also reports that the heart tissue coenzyme Q_{10} concentration has been increased[32,38] following its oral administration. Also, in kidney and brain tissues we did not observe change in tissue concentration of Q_9 or Q_{10} due to supplementation, which agrees with other studies.[19,34,35] Unlike cholesterol, another lipid from the mevanolate pathway, liver secretes only a small amount of coenzyme Q into the circulatory system.[34] In addition, this secreted coenzyme Q is not distributed to organs, but associates with LDL and VLDL.[39] The fact that supplementation did not result in an increase in heart or kidney tissues confirms these previous observations. Furthermore, the lack of increase in brain tissue Q_{10} concentration in rats or CSF concentration in humans suggests that exogenous Q_{10} does not cross the blood-brain barrier and does not participate in the regulation of coenzyme Q concentration in brain.

27.4.3 Comments on the Methodology of Coenzyme Q Measurements

It is well known that coenzyme Q exists in tissues in both oxidized and reduced form and that the distribution of these two redox states differs between different tissues.[40] There are many different methods that are based mostly on HPLC nowadays, developed for the detection of coenzyme Q in tissues and plasma.[26,31,40–44] Many of these[26,40,43] permit one to determine both oxidized and reduced forms of coenzyme Q simultaneously. The sensitivity of these methods has been reported to be high and even p-molar amounts can be routinely detected.[26]

We measured the total coenzyme Q concentrations. The determination was done with HPLC with an electrochemical detection based on the method of Lang et al.[26] In our hands, the method was not sensitive enough for the detection of both oxidized and reduced forms of coenzyme Q. Therefore, the total coenzyme Q_9 and Q_{10} content in tissues after reducing the sample with sodium dithionite was measured. On the other hand, since Q_{10} could be considered to represent exogenous coenzyme Q incorporation into rat tissues, the measurement of total coenzyme Q content in tissues proved to be suitable for observing this incorporation.

27.5 CONCLUSIONS

Several reports indicate that orally administered coenzyme Q_{10} is readily absorbed in both men and rats and its plasma concentration increases significantly in as little as 2-weeks after supplementation in men. However, it seems that coenzyme Q_{10} does not pass the blood-brain barrier: we have shown that in rats, brain tissue concentration, and in men, CSF concentration did not increase due to supplementation. Also, it did not accumulate in rats in any tissue other than liver.

Contrary to our expectation, the lifelong supplementation did not prolong the lifespan of either rats or mice. The growth and the gain of weight were similar in both supplemented and control rat groups. With an increasing age in rats, plasma and liver coenzyme Q_{10} concentrations increased up to 18 months and then decreased. In the liver, Q_9 concentration behaved similarly. In the heart, coenzyme Q_{10} concentration increased up to 12 months. No differences during aging were observed in kidney or brain tissues. Lifelong coenzyme Q_{10} supplementation increased Q_{10} concentration in liver. In old rats, Q_9 concentration was also increased in liver in the supplemented group. Lifelong supplementation had no effect on coenzyme Q_9 or Q_{10} concentrations in the heart, kidney, or brain tissues. There was no reduction of the lipopigment accumulation in either adrenal cortex or superior cervical ganglia due to lifelong coenzyme Q_{10} supplementation in rats. Also, no differences between groups were observed in macroscopic or microscopic evaluation of the rat tissues.

In conclusion, our studies showed that orally administered coenzyme Q_{10} was followed by an increase in plasma and liver coenzyme Q_{10} concentrations, while other tissues were not affected. This indicates that tissues synthesize de novo the coenzyme Q they need. The lifelong coenzyme Q_{10} supplementation of rats and mice was not followed by an increased life span or decreased morbidity. Also, the autopsies and histological examination of tissues showed no differences between the groups.

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