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# HYPOTHESIS

# UBISEMIQUINONES OF THE MITOCHONDRIAL RESPIRATORY CHAIN DO NOT INTERACT WITH MOLECULAR OXYGEN

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The present investigation deals with the suggested role of redox-cycling ubisemiquinones in mitochondrial $O_2^-$ generation. Due to the functional complexity of electron-transferring ubiquinones in the respiratory chain, model experiments were designed to study whether ubisemiquinones will directly react with oxygen, thereby generating $O_2^-$ radicals. Based on the fact that mitochondrial ubiquinone was reported to operate in an aprotic surrounding of the inner mitochondrial membrane, the reactivity of ubisemiquinones with oxygen was tested in water-free acetonitrile. Our results prove that autoxidation of ubisemiquinones requires the addition of protons to the non-polar reaction system. An experimental evaluation of the validity of this finding with respect to mitochondrial ubiquinones is impeded by the biochemical role that oxygen plays in the establishment of ubisemiquinone populations. To differentiate between a possible direct interaction of oxygen on redox-cycling ubisemiquinones and this indirect biochemical $O_2$ effect, we have successfully introduced ferricyanide instead of oxygen to establish mitochondrial ubisemiquinone pools. Ubisemiquinones in this reaction system were not susceptible to oxygen and no $O_2^-$ radicals were released unless the inner mitochondrial membrane was protonated by toluene pretreatment. Since the inner mitochondrial membrane is normally not permeable to protons (which is a prerequisite of the chemiosmotic theory of energy conservation) based on our experiments we can exclude the involvement of redox-cycling ubisemiquinones in mitochondrial $O_2^-$ generation.

KEY WORDS: Mitochondria, ubisemiquinones, superoxide radicals.

## INTRODUCTION

Apart from cytochrome oxidase, redox cycling ubiquinone (Q) was also suggested to directly interact with molecular oxygen.[1–5] This assumption was based on the finding that under certain conditions of respiration also uni- and divalently reduced oxygen ($O_2^-$; $H_2O_2$) were released besides water (the tetravalent reduction product of cytochrome oxidase). Hydrogen peroxide was found to originate from dismutating $O_2^-$ while the latter was reported to stem from a divergent single electron transfer from redox cycling ubisemiquinone ($SQ^{\cdot}$).

The following set of experimental observations were considered as evidences for this assumption. Trumpower and Simmons have reported a SOD-sensitive increase in cyt *c* reduction when electron flow from complex II and recycling of electrons into the

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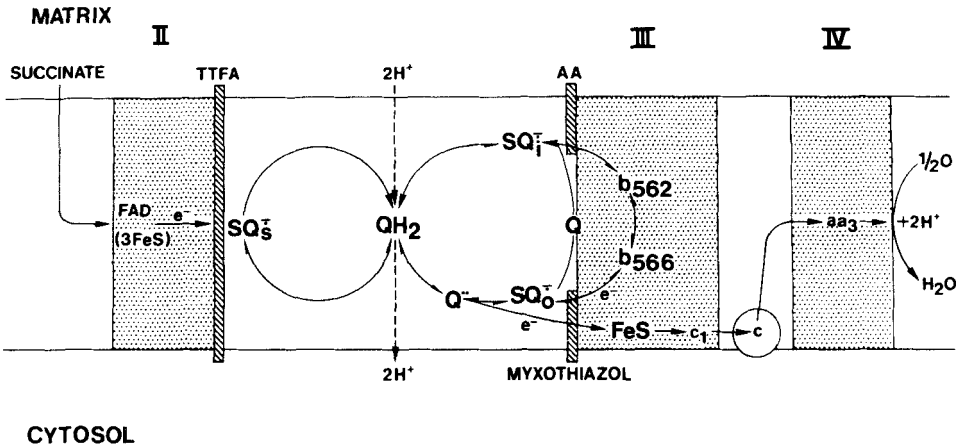


FIGURE 1 Proposed scheme of ubiquinone-mediated $e^-$ transfer between succinate dehydrogenase of complex II and the $bc_1$ complex (complex III).[20] Electrons from complex II are carried through the ubiquinol-pool ($QH_2$) and the first $e^-$ is linearly transferred to the Rieske iron sulfur protein (FeS) following binding close to the outer phase of the inner mitochondrial membrane at complex III. The second $e^-$ of ubisemiquinone formed at center out ($SQ_0$) is then recycled into the $QH_2$-pool after equilibration with *b*-type-cytochromes completing a loop of $e^-$-cycling (Q-loop). Associated with the linear transfer of one electron, two protons are extruded from the matrix to the cytosol. All redox-cycling $SQ^{\cdot}$ exist in the anionic form and are stabilized through binding to the respective complexes.

ubiquinone-loop were inhibited by the addition of thenoyltrifluoroacetone (TTFA) and antimycin A (AA).[4] The authors have interpreted their finding in terms of a steady state increase of autoxidizing ubisemiquinone. The resulting generation of $O_2^{\cdot-}$ radicals was suggested to account for the reduction of cyt *c* via a short circuit.

This interpretation does not fit with the observation that in the presence of AA and TTFA, $SQ^{\cdot}$ population was below the normal level.[6,7] The observed decrease of steady state concentrations of mitochondrial $SQ^{\cdot}$ is due to destabilization of $SQ^{\cdot}$ pools following their displacement from complex II and III respectively rather than dissipation of $SQ^{\cdot}$ through continuous autoxidations. $O_2^{\cdot-}$ formation in that case is more likely to originate from autoxidation of a component of complex II, since all $e^-$ carriers of this complex exhibit highest states of reduction when electrons are hindered from being regularily transferred to ubiquinone.[8]

However, other lines of experimental evidence exist in the literature which also support redox cycling Q as an alternative site of direct oxygen interaction during respiration: e.g., $H_2O_2$ release from decomposing $O_2^{\cdot-}$ was reported to be inhibited after removal of Q from mitochondria, but was reestablished after reincorporation of added Q,[5] and myxothiazol, which prevents the existence of ubisemiquinone at its outer binding center to the $bc_1$ complex (See Figure 1) was also found to inhibit mitochondrial $O_2^{\cdot-}$ formation.[9,10]

Objections regarding the validity of conclusions drawn from these experiments become evident when considering that inhibition of Q-redox-cycling will also prevent electron transfer to other carriers of the respiratory chain possibly acting as reductants of molecular oxygen (see Figure 1). Further arguments against the role of redox-cycling Q in mitochondrial $O_2$-activation come from thermodynamic considerations of this lipophilic electron carrier. Autoxidation is not likely to occur as long as $SQ^{\cdot}$ is

operating in the anhydrous surrounding of the phospholipid bilayer. Under these conditions we have calculated that free energy changes associated with autoxidation of $SQ^{\cdot}$ will not allow this reaction to proceed spontaneously. On the other hand, a direct interaction of molecular oxygen with redox-cycling Q would directly impair energy-linked respiration by disturbing the control of divergent electron flux thereby inhibiting the proton-motive ubiquinone-cycle (see Figure 1).

The complicated nature of the proton motive ubisemiquinone cycle impedes the design of a conclusive experimental approach to study possible interactions of molecular oxygen with redox-cycling Q in mitochondria. Thus, we have reduced this problem to the general question as to whether isolated ubisemiquinone will react with molecular oxygen when existing in a lipophilic surrounding, as was reported to be the case when operating in the inner mitochondrial membrane. Our results indicated the requirement of protons for this type of reaction. This also infers that the lipophilic character of the inner mitochondrial membrane protects redox cycling $SQ^{\cdot}$ from a direct chemical interaction with oxygen. To prove this experimentally, we have developed a methodological approach which permits the study of the effect of oxygen on steady state concentrations of mitochondrial $SQ^{\cdot}$ in the absence and presence of protons.

## MATERIALS AND METHODS

Potassium superoxide ($KO_2$) came from Fluka AG, Buchs, Switzerland. Toluene (p.a.), 2,5,8,15,18,21-hexaoxatricyclo-[20.4.0.$0^{9.14}$]-hexacosane (crown ether), potassium hexacyanoferrate-(III) ($K_3Fe(CN)_6$) ("ferricyanide") and acetonitrile (gradient grade) were purchased from Merck, Darmstadt, Germany. Acetonitrile was purified from adjectant water with a molecular sieve (3 Å, Merck). 2,3-Dimethoxy-5-methyl-1,4-benzoquinone (coenzyme $Q_0$), myxothiazol, Cu–Zn SOD, N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES) and antimycin A were from Sigma, Chemical Co, St. Louis, USA. Other biochemicals and chemicals were purchased from Boehringer GmbH, Mannheim, Germany and Merck, Darmstadt, Germany. Mitochondria were prepared from hearts and livers of male Sprague Dawley rats weighing between 250 and 300 g. Heart mitochondria were prepared according to Ref.[11] Liver mitochondria were isolated as described in Ref.[12] Respiratory control values and P/O ratios were determined by measuring oxygen consumption rates under state 4 and state 3 conditions using a micro-Clark type electrode designed in the laboratory of Prof. M. Klingenberg, Munich, Germany.

Dimethyl-1-pyrroline-N-oxide (DMPO) from Aldrich-Europe, Belgium was purified and stored as described elsewhere.[7] Due to the low level of mitochondrial ubisemiquinones, detection of these radical species required high concentrations of mitochondria in the ESR sample cell. Since the weight of rat hearts is one tenth of normal rat livers, we decided to prepare liver mitochondria for ubisemiquinone investigations due to the much higher yield from this organ. Preinvestigations and earlier publications[7] convinced us that redox-cycling ubisemiquinones behave identically in both types of mitochondria. The ESR experiments were carried out in a BRUKER ER 200 D-SRC 9/2.7 spectrometer operating at 9.6 GHz with 100 kHz modulation frequency equipped with a variable temperature unit. All samples were rapidly frozen in liquid nitrogen in order to inhibit further reaction and were measured at $-30$°C in order to prevent power saturation which was observed at 77 K.

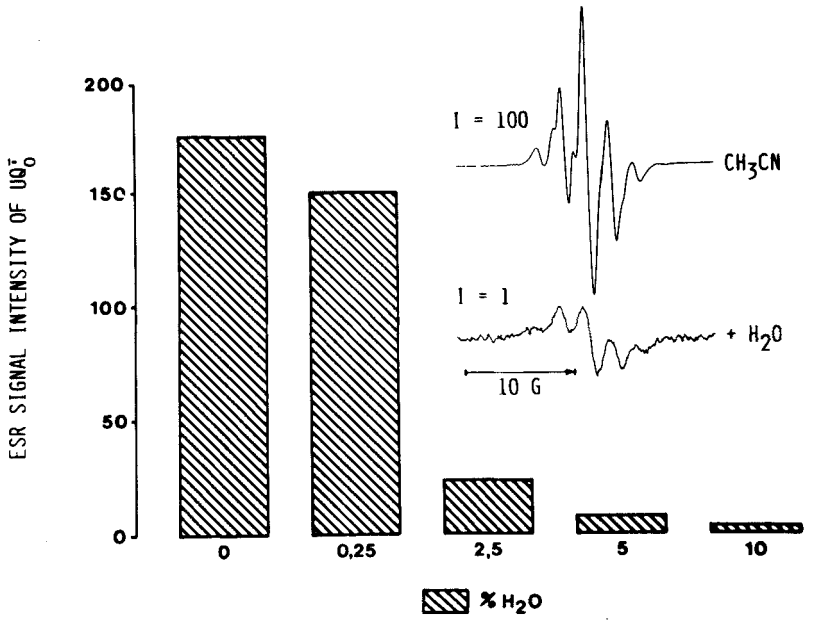


FIGURE 2 ESR signal intensity of the ubiquinone$_0$-radical ($UQ_0^{\cdot}$) at different $H_2O$ concentrations in arbitrary units. The concentration of $UQ_0$ in acetonitrile was 20 mM. The spectrometer settings were: scan range, 40 G; modulation amplitude, 0.2 G; receiver gain, $5 \times 10^3$; microwave power, 2 mW; time constant, 0.65 s; scan rate, 14.3 G/min. The inset shows the comparison of the ESR signal intensity in the absence and presence of water, the intensity of the upper spectrum has to be multiplied by a factor of 100.

## RESULTS

### *Reactivity of Ubisemiquinones with Oxygen in an Aprotic and Proton-containing Environment*

Ubiquinone ($Q_0$) was dissolved in water-free acetonitrile and mixed with $KO_2$. Superoxide radicals released from decomposing $KO_2$ disappeared from the medium within 90 s (tested with DMPO; exp. not shown). This was also the time period required to start running ESR spectra shown in Figure 2. The split ESR signal obtained in acetonitrile ($CH_3CN$) revealed hyperfine interactions of the unpaired electron with protons associated with the benzoquinone ring of $Q_0$. In the absence of $Q_0$, ESR spectroscopy remained silent. Thus the ESR signal observed clearly indicated the existence of $SQ_0^{\cdot}$ in our reaction medium generated according to Eq. (1)

$$Q_0 + O_2^{\cdot -} \leftarrow SQ^{\cdot -} + O_2. \qquad (1)$$

The presence of air oxygen did not affect the equilibrium of Eq. (1). However, protonation of the reaction system upon the addition of water stimulated the backward reaction, since $SQ_0^{\cdot}$ related ESR signals became smaller the more water was mixed to the solvent (Eq. (2)).

$$Q_0 + HO_2^{\cdot} \xrightarrow[H^+]{} SQ_0^{\cdot -} + O_2. \qquad (2)$$

Solvent changes were also reflected by altered hyperfine splitting of the remaining $SQ_0^{\cdot}$

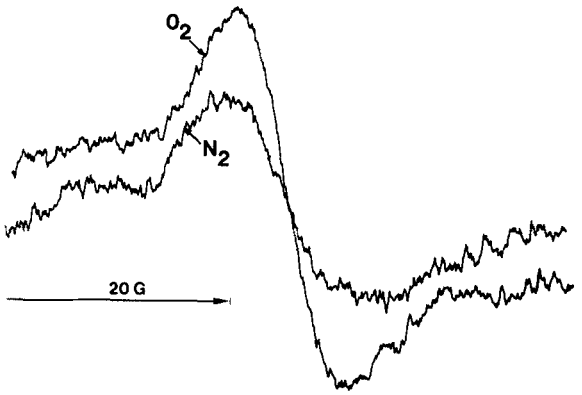


FIGURE 3 Oxygen-induced increase of mitochondrial ubisemiquinone population. The incubation mixture contained 3.2 mg/ml RLM, 24 mM fumarate/succinate 1:5, and 7.3 mM phosphate. The ESR spectra were taken in the presence ($O_2$) and absence ($N_2$) of oxygen under otherwise identical conditions. Oxygen was mixed to the reaction system by bubbling with pure oxygen and removed by bubbling with ultrapure $N_2$ at ambient temperature over a period of 2 min. The spectrometer settings were: scan range, 50 G; modulation amplitude, 5 G; receiver gain, $5 \times 10^5$; microwave power, 1 mW; time constant, 2.6 s; scan rate, 9 G/min, 5 scans.

related ESR spectra, and broadening indicated possible interactions with $O_2^{\cdot-}$ radicals formed. These findings demonstrate the requirement of protons for a reaction of oxygen with ubisemiquinone.

### *The Effect of Oxygen on Steady State Concentration of Mitochondrial Ubisemiquinones*

Relaxation properties and spin coupling to neighbouring flavin semiquinones or iron–sulfur centres require particular experimental care in order to detect mitochondrial $SQ^{\cdot}$.[13] When poising mitochondria at low succinate/fumarate ratios ($E_h \approx +50$ mV), redox potentials established favour thermodynamically the formation and detection of redox-cycling $SQ^{\cdot}$ by means of ESR even at ambient temperature[6] (see Figure 3).

Since ubisemiquinones involved in mitochondrial $e^-$ transfer were unequivocally reported to operate in the aprotic phase of the inner membrane,[14] dioxygen was not expected to affect the population of redox-cycling $SQ^{\cdot}$. However, oxygenation of an anaerobic mitochondrial suspension was found to cause a drastic increase in $e^-$-transferring $SQ^{\cdot}$ (Figure 3). This observation was suggested to reflect bioenergetic effects of $O_2$ on the redox states of mitochondrial $e^-$-carriers. Based on the functional properties of the proton-motive Q-cycle, one may assume that in the absence of oxygen, the mitochondrial $SQ^{\cdot}$ population is at a low level although other $e^-$-carriers including the Rieske iron sulfur protein ($FeS_R$) (which is the oxidant of ubiquinol) exhibit highest states of reduction. The complete reduction of $FeS_R$ prevents oxidation of ubiquinol to $SQ^{\cdot}$ and consequently inhibits $e^-$ flow through the Q-cycle (see Figure 1). Aerobic respiration keeps $FeS_R$ more or less oxidized so that $SQ^{\cdot}$ formation from ubiquinol can proceed. Thus, differentiation of this indirect oxygen effect (which increases steady state concentrations of $SQ^{\cdot}$) from a direct oxygen effect (which should rather decrease $SQ^{\cdot}$ concentration by autoxidation) is not possible under normal conditions of respiration. We therefore tested whether non-physiological

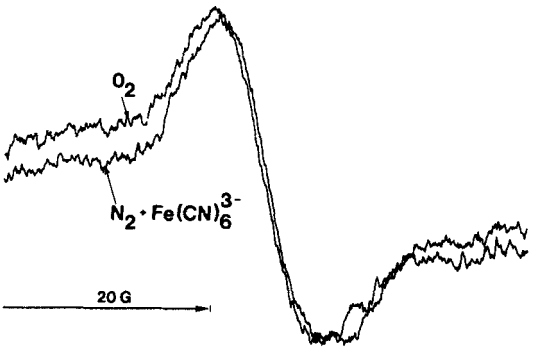


FIGURE 4 The effect of ferricyanide on mitochondrial ubisemiquinone population. The incubation mixture contained 3.2 mg/ml RLM, 24 mM fumarate/Succinate 1 : 5, and 7.3 mM phosphate. The ESR spectra were taken in the presence ($O_2$) and absence ($N_2$ + 12 mM $Fe(CN)_6^{3-}$) of oxygen. The spectrometer settings were the same as in Figure 3.

$e^-$-acceptors can be used to allow one-$e^-$ transfer from ubiquinol to its physiological oxidant despite the absence of oxygen.

*Replacement of Oxygen by Ferricyanide in Keeping Redox-cycling Ubisemiquinone Operating*

Figure 4 shows that ferricyanide has the same effect on mitochondrial $SQ^{\cdot}$ population as oxygen. Ferricyanide is known to directly induce oxidation of cyt *c*, thereby necessarily running the Q-cycle in the same way as described for oxygen (Figure 3). Equilibration of this anaerobic reaction system with oxygen did not affect the intensity of the $SQ^{\cdot}$ related ESR-signal (Figure 5). This was not unexpected assuming that redox-cycling Q operates within the lipophilic phase of the inner mitochondrial membrane to which protons do not have access.

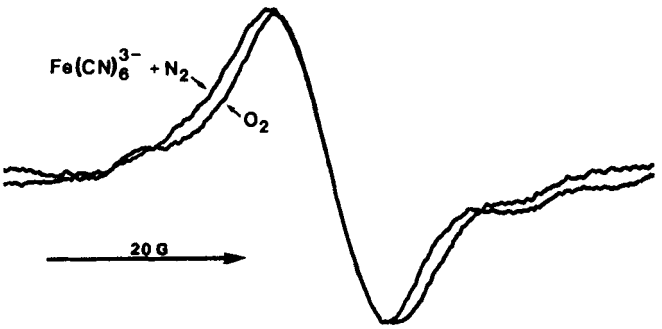


FIGURE 5 The effect of $O_2$ on redox cycling mitochondrial ubisemiquinone in the presence of ferricyanide. The incubation mixture contained 8.7 mg/ml, RLM, 26 mM fumarate/Succinate 1 : 5, and 8 mM phosphate. The ESR spectra were taken in the presence ($O_2$) and absence ($N_2$ + 13 mM $Fe(CN)_6^{3-}$) of oxygen. The spectrometer settings were: scan range, 100 G; modulation amplitude, 5 G; receiver gain, $1 \times 10^5$; microwave power, 10 mW; time constant, 0.65 s; scan rate, 36 G/min, 15 scans.

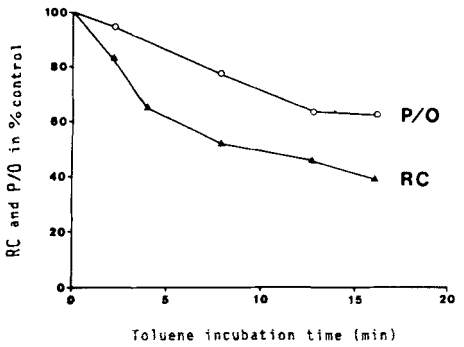


FIGURE 6 The effect of toluene incubation time on the proton permeability of the inner mitochondrial membrane as evaluated from respiratory control values (RC) and ATP/oxygen ratios (P/O). Absolute RC values: 100% = 6.40; absolute P/O values: 100% = 2.40. 0.3 mg of freshly isolated heart mitochondria were suspended in 0.6 ml of the isolation buffer (300 mM sucrose, 20 mM triethanolamine-HCl, 2 mM EDTA; pH 7.4) supplemented with 0.3 mg of bovine serum albumin. 5 $\mu$l of toluene was added and the incubation system was gently stirred under air–oxygen at 25°C. Since the incubation procedure with toluene was directly performed in the vessel in which the Clark-type electrode was inserted the various incubation times were stopped upon starting state 4 respiration with glutamate/malate (200 $\mu$M final conc.), followed by ADP (416 $\mu$M final conc.) and $P_i$ (4.1 mM final conc.) addition to run state 3 respiration.

### *Permeability Changes of the Normally Proton-tight Inner Mitochondrial Membrane to Protons*

Pretreatment of isolated mitochondria with toluene affected the inner membrane in such a way that energy coupling (ATP/O) and respiratory control values (RC) decreased the longer they were exposed to this lipophilic compound (Figure 6). In the absence of toluene, RC and P/O values were only slightly changed during the time period studied (not shown). RC and P/O ratios are indirect indicators of the existence of vectorial membrane proton translocation linked to respiration. As a consequence, protons are accumulated at the cytosolic face of the inner mitochondrial membrane. This proton gradient is used to run oxidative phosphorylation.[15] Thus, decreased ATP formation reflected by decreased P/O values indicate insufficient proton gradients due to rediffusion of extramitochondrial protons through the inner membrane or as a consequence of insufficient transmembraneous proton translocation, e.g., in the case of inhibition of mitochondrial respiration. The latter assumption can be ruled out since respiratory activities of toluene pretreated mitochondria were not inhibited (exp. not shown). Assuming that toluene pretreatment makes the membrane proton permeable, it was of interest to study whether $SQ^{\cdot}$ of toluene-pretreated mitochondria becomes susceptible to oxygen according to Figure 2. The results documented in Figure 7 clearly demonstrate that in toluene pretreated mitochondria, the $SQ^{\cdot}$-related ESR signal decreased when oxygen was present. Oxygen effects were found to become clearer the more RC and P/O values decreased. A direct interaction of oxygen with the redox-cycling $SQ^{\cdot}$ is expected to generate $O_2^{\cdot-}$ radicals according to Eq. (2). Figure 8 compares conditions required to initiate $O_2^{\cdot-}$ release from untreated and toluene pretreated mitochondria. Untreated RHM (Figure 8A) cannot be made to produce any $O_2^{\cdot-}$ radicals unless respiration was inhibited by the addition of AA ("classical conditions"). As earlier reported, small amounts of $O_2^{\cdot-}$ radicals can be detected

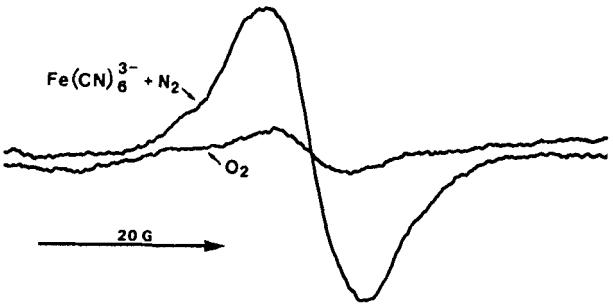


FIGURE 7 The effect of membrane protonation on $O_2$ susceptibility of redox cycling mitochondrial ubisemiquinone. 3 mg of freshly isolated RLM were suspended in 3 ml of the isolation buffer at ambient temperature. 100 $\mu$l of toluene were added and the incubation system was gently stirred under air–oxygen. After 4 min, the incubation was stopped by diluting with the isolation buffer and subsequent sedimentation at 8000 g. The washing procedure was repeated two more times to ESR measurements. The reaction mixture contained 21.7 mg/ml RLM of the resuspended pellet, 26 mM fumarate/succinate 1 : 5, and 8 mM phosphate. The ESR spectra were taken in the presence ($O_2$) and absence ($N_2$ + 13 mM $Fe(CN)_6^{3-}$) of oxygen. The spectrometer settings were the same as in Figure 5.

despite the presence of matrix bound SOD, indicating a vectorial release of $O_2^{\cdot-}$ across the inner membrane.[16]

Myxothiazol caused an almost total inhibition confirming the involvement of the $SQ^{\cdot}_{out}$-cyt *b* section in univalent oxygen activation.[10]

Mitochondria pretreated with toluene began generating $O_2^{\cdot-}$ radicals shortly after

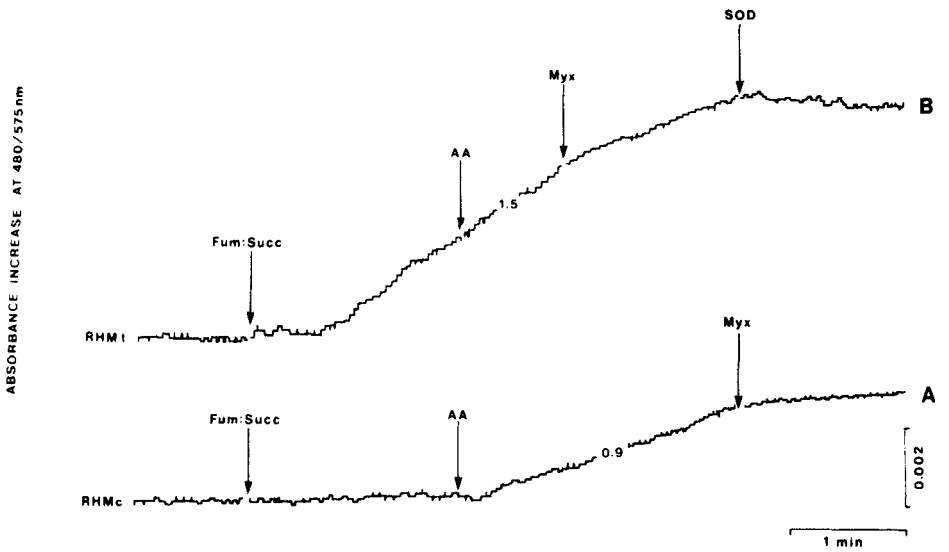


FIGURE 8 The effect of toluene pretreatment on superoxide generation from rat-heart mitochondria poised with the same fumarate/succinate ratio as used in figures 3–5 and 7. $O_2^{\cdot-}$ formation rates were calculated from SOD-sensitive cooxidation rates of epinephrine to adrenochrome measured in a dual wavelength spectrophotometer (Shimadzu 2000) at 480 nm as in Ref. 16. The numbers associated with the curves represent nmols of $O_2^{\cdot-}$ min/mg of mitochondrial protein. $RHM_c$ = control; $RHM_t$ = toluene pretreated rat heart mitochondria.

running respiration with the same fumarate/succinate ratio used before. AA was not required and had no stimulating effect. Myxothiazol sensitivity towards $O_2^{\cdot-}$ generation was also observed, indicating the necessity of an intact $e^-$-cycling through the Q-loop. $O_2^{\cdot-}$ generation rates detected in the extramitochondrial space were significantly higher than in the control experiments observed after the addition of AA.

## DISCUSSION

### *Ubisemiquinones Operating in Intact Mitochondria are Unlikely to Account for Mitochondrial $O_2^{\cdot-}$-Release*

The susceptibility of $e^-$ transferring mitochondrial $SQ^{\cdot}$ to oxygen was concluded from the observation of $O_2^{\cdot-}$ release associated with $SQ^{\cdot}$ formation from ubiquinol.[2,4,9] Inhibition of the $e^-$-transferring Q-cycle by removing Q from the inner membrane[5] or by the use of myxothiazol[9] were taken as evidence supporting this assumption, since $O_2$-activation disappeared under these conditions. The establishment of an efficient redox couple which may function as an $O_2^{\cdot-}$ generator requires a permanent shuttle of reducing equivalents to the respective reductant. As shown in Figure 1, cyt *b* also takes part in $e^-$ recycling of the Q-loop. This means, that inhibition of the Q-cycle also causes inhibition of redox-cycling cyt *b*. Thus, both, $e^-$ transferring $SQ^{\cdot}$ and cyt *b* have to be considered as possible $O_2^{\cdot-}$ sources in mitochondria. Low potential cyt *b* was reported to exhibit thermodynamic properties permitting autoxidation under conditions of energy-linked respiration.[10] The present paper indirectly supports this assumption by excluding a direct reaction of $O_2$ with $SQ^{\cdot-}$ on thermodynamic grounds. This was demonstrated by the stability of $SQ_0^{\cdot-}$ formed from $Q_0$ in the presence of oxygen in water-free acetonitrile (Figure 2). Oxygen was without effect on the equilbrium of the reaction. This was in accordance with free energy changes calculated on the basis of one-$e^-$ redox potentials of $Q/SQ^{\cdot-}$ and $O_2/O_2^{\cdot-}$ published for aprotic solvents.[17–19]

### *Permeability of the Inner Mitochondrial Membrane to Protons makes $SQ^{\cdot}$ Directly Susceptible to Oxygen*

The equilibrium shift of the reaction in favour of autoxidation of $SQ^{\cdot}$ which was observed after protonation of the medium was in harmony with exergonic energy changes calculated for a reaction of $SQ^{\cdot}$ with oxygen. The examination of this finding with respect to autoxidation behaviour of $SQ^{\cdot}$ operating in the inner mitochondrial membrane raised two major experimental problems. One is the fact that oxygen is required to establish $SQ^{\cdot}$ formation of the Q-cycle (see Figure 1), while on the other hand it was our aim to see whether or not oxygen can also directly oxidize $e^-$ transferring $SQ^{\cdot}$ of the respiratory chain. This conflicting problem was solved by replacing the biochemical function of oxygen in maintaining $SQ^{\cdot}$ generation of the Q-cycle by ferricyanide (see Figure 4).

The fact that the addition of $O_2$ to this system did not affect steady state concentrations of $SQ^{\cdot}$ was in harmony with our *in vitro* experiments where $SQ^{\cdot}$ radicals remained insensitive to $O_2$ in the absence of protons. The lack of oxygen effect on steady state concentrations of mitochondrial $SQ^{\cdot}$ (which operate in the proton impermeable mitochondrial membrane) does not exclude autoxidation, assuming higher rate constants for regeneration of $SQ^{\cdot}$ than for a reaction with oxygen.

However, this consideration can be ruled out since $O_2^{\cdot-}$ radicals which should be formed in this case were not detected. In contrast, toluene pretreated mitochondria were found to exhibit both a decrease of mitochondrial $SQ^{\cdot}$ population in the presence of oxygen and the simultaneous formation of $O_2^{\cdot-}$ during respiration. These observations infer that rate constants of $SQ^{\cdot}$ autoxidation exceed those of $SQ^{\cdot}$ recovery from ubiquinol. Futhermore, in analogy to the proton effect of $SQ^{\cdot}$ autoxidation demonstrated in the pure chemical system (see Figure 2), these observations strongly suggest that toluene pretreatment changes mitochondrial membranes in such a way that $e^-$ carrying $SQ^{\cdot}$ become accessible to protons. This was the second experimental problem to be solved. P/O and respiratory control values which normally reflect the resistance of mitochondria to transmembraneous proton conductance indicated a dose-related loss of this property following toluene pretreatement (see Figure 6).

Toluene-induced collapse of the transmembraneous proton gradient is not likely to occur in the same way as known from classical uncouplers such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 2,4-dinitrophenol. These protonophores reconduct extramitochondrial protons through a pK-dependent proton translocation. Thus, in that case protons will not have access to redox-cycling $SQ^{\cdot}$ and consequently autoxidation of $SQ^{\cdot}$ is not to be expected on thermodynamic grounds. This was confirmed by the lack of $O_2^{\cdot-}$ release in the presence of these protonophores (exp. not shown). Since uncoupling by toluene is linked both to a decrease of redox-cycling $SQ^{\cdot}$ and a simultaneous $O_2^{\cdot-}$ release from mitochondria, the collapse of the transmembraneous proton gradient most likely occurs via a non-specific proton leak pathway. This allows a collision of rediffusing protons with redox-cycling $SQ^{\cdot}$ and subsequent autoxidation of this $e^-$ carrier. Autoxidation of $e^-$ transferring $SQ^{\cdot}$ is not expected to occur under *in vivo* conditions since proton impermeability of the inner mitochondrial membrane is a prerequisite of energy conservation.[15] Proton movements across the inner mitochondrial membrane associated with respiration occurs through distinct anhydrous proton channels. Thus, the establishment of the $\Delta pH$ during respiration will not provide thermodynamic conditions for $SQ^{\cdot}$ autoxidation. Indirect evidences for this assumption emerge from our recent observations that also (patho)-physiological impairments of the resistence of the mitochondrial membrane to proton penetration is associated with $O_2^{\cdot-}$ stimulation (papers in preparation).

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