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Studies on the stabilized ubisemiquinone species in the succinate–cytochrome *c* reductase segment of the intact mitochondrial membrane system

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1. Evidence is presented for the presence of a stable ubisemiquinone pair in the vicinity of iron–sulphur centre S-3, based on its thermodynamic and spin relaxation properties. 2. These semiquinones are coupled by dipolar interaction; quantitative analysis of the signals of the spin-coupled semiquinones (at pH 7.4) gives midpoint redox potentials E_1 (oxidized to semiquinone state) and E_2 (semiquinone to fully reduced state) of 140 and 80 mV, respectively, for individual ubiquinones. 3. Values of pK_S (pK of the semiquinone form) below 6.5 and pK_R (pK of the fully reduced ubiquinone) of about 8.0 or above were estimated from the pH-dependence of the midpoint potentials of the spin coupled signals. Thus the ubisemiquinone associated with succinate dehydrogenase (designated as SQ_s) functions mostly in the anionic form in the physiological pH range. 4. Thenoyltrifluoroacetone, a specific inhibitor of the succinate–ubiquinone reductase segment of the respiratory chain, destabilized the intermediate redox state; thus it quenches both the $g = 2.00$ signal of ubisemiquinone (SQ_s) and split signals from the spin coupled pair. This inhibitor has no significant effect on another bound ubisemiquinone species present in the cytochrome bc_1 region (designated as SQ_c). 5. The possible function and location of these stabilized ubisemiquinone species were discussed in connection with Site-II energy transduction.

The final step in electron transfer from succinate to Q is believed to involve 'Hipip-type' iron–sulphur centre S-3 in succinate dehydrogenase (Beinert *et al.*, 1975; Ohnishi *et al.*, 1976). At intermediate redox states, but not in the fully oxidized or reduced state, the e.p.r. spectrum of centre S-3 in mitochondrial membrane preparations is accompanied by partially overlapping signals at $g = 2.04$, 1.99 and 1.96 (Ruzicka & Beinert, 1975). This suggests that these signals are associated with free radical states of the $n = 2$ electron-transfer components such as flavin or Q. Two very important findings were reported by Ruzicka & Beinert (1975): (i) these multiple signals arise from a spin–spin interaction because their field position is independent of the frequency of the applied microwaves; (ii) ubisemiquinone is one of the interacting species because these signals disappear upon depletion of Q from the mitochondrial membrane and reappear upon replenishment of Q. Initially, the accompanying signals were suggested to arise from a spin–spin interaction between ubisemiquinone and centre S-3 spins, because of the concurrent appearance of their signals and of their very short relaxation times, typical of transition metals. The same group, in collaboration with Sands and his colleagues, subsequently performed computer-simulation studies of the e.p.r. spectrum of Complex II that was trapped kinetically at an intermediate redox state (Ruzicka *et al.*, 1975). A somewhat better fit was obtained for spectra simulated as a ubisemiquinone–semiquinone (either QH· or flH·) spin–spin interaction overlapped with non-interacting S-3 signals, rather than a spin–spin interaction between ubisemiquinone and centre S-3.

Ingledew and colleagues (Ingledew & Ohnishi, 1975; Ingledew *et al.*, 1976) raised questions about

Abbreviations used: Q, ubiquinone; QH·, protonated form of ubisemiquinone; $Q^{\cdot-}$, deprotonated form of ubisemiquinone; Q_s and SQ_s, ubiquinone and ubisemiquinone associated with succinate dehydrogenase; Q_c and SQ_c, ubiquinone and ubisemiquinone in the cytochrome bc_1 region; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; fl, flavin; TTFA, thenoyltrifluoroacetone.

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the direct spin–spin interaction between ubisemiquinone and centre S-3, based on a spin-concentration analysis of centre S-3 and the accompanying signals, with mitochondrial systems poised at various redox potentials. They also proposed ubisemiquinone rather than flavin free radical as the interacting partner, on the basis of the measured midpoint potentials.

Thenoyltrifluoroacetone (TTFA) is a potential metal-chelating agent which inhibits almost completely succinate–ubiquinone reductase (Baginsky & Hatefi, 1969) or succinate oxidase (King, 1966) activity, but in micromolar concentrations it does not inhibit electron transfer from succinate to artificial redox dyes. Ackrell *et al.* (1977) reported that the reduction rate of this centre by succinate is not affected by TTFA, whereas the re-oxidation rate of this centre by a Q analogue was greatly decreased. Thus the inhibition site was assigned between centre S-3 and the Q pool.

Ingledew and colleagues reported that TTFA removes e.p.r. signals arising from spin–spin interaction between ubisemiquinone pairs at concentrations comparable with those inhibiting succinate oxidation (Ingledew & Ohnishi, 1977; Ingledew *et al.*, 1977). They also demonstrated that the maximum signal intensity, lineshape and relaxation behaviour of centre S-3 was not significantly altered by TTFA, but the midpoint potential of centre S-3 decreased in the absence of spin–spin interaction between the ubisemiquinone pair.

Analysis of the microwave-power dependence of the $g = 2$ signal revealed a heterogeneous population of ubisemiquinone; a part of the Q pool showed abnormally rapid relaxation, seen as the non-saturated fraction at 100mW. This cross-relaxation of ubisemiquinone spin by rapidly relaxing centre S-3 was completely perturbed by a low concentration of TTFA (Ingledew *et al.*, 1977). Ruuge & Konstanchinov (1976) had previously detected anomalous saturation behaviour of one of the Q pools. They reported independently that this unsaturable $g = 2$ signal was lost in the presence of TTFA. Concomitantly, e.p.r. signals, arising from spin–spin interaction, observed at $g = 2.04$ and 1.99 were completely suppressed (Konstanchinov & Ruuge, 1977*a*). Sensitivity to TTFA can be used as a useful marker for the $g = 2$ free radical signal arising from a Q pool that is in the proximity of succinate dehydrogenase.

In the present paper we report the results of the potentiometric titration of ubisemiquinone signals as well as low-temperature $Q^{\cdot-}Q^{\cdot-}$ interaction signals, monitored directly in beef heart submitochondrial particles. Response to TTFA was used to distinguish the different Q species in the mitochondrial membrane; one in the vicinity of succinate dehydrogenase (designated here as SQ_s) and other in the cytochrome bc_1 region (designated as SQ_c). The pH-dependence of the titrations of the split signals from the spin-coupled Q pair was used to determine the protonation states of reduced forms of Q and to estimate pK_S (pK value of the ubisemiquinone form) and pK_R (pK value of the fully reduced ubiquinone). The effects of magnetic and electrostatic interactions are discussed.

Parts of this work were presented at the International Symposium of Membrane Bioenergetics at Spetsai, Greece (Ohnishi *et al.*, 1977).

Materials and methods

Beef heart mitochondria and submitochondrial particles were prepared as described by Löw & Vallin (1963). Potentiometric titrations of the suspension of submitochondrial particles were conducted anaerobically in the presence of several redox dyes as described previously (Wilson *et al.*, 1970; Dutton, 1971). Detailed conditions are described individually in the Figure legends. The samples were rapidly frozen in e.p.r. tubes by immersing them into methylcyclohexane/isopentane (1:5, v/v) at 81K. Temperature of e.p.r. measurements was controlled by a variable-temperature cryostat (Air Products LTD-3-110). The temperature (below 50K) was monitored with an Allen–Bradley carbon resistor directly below the sample. Sample temperature above 170K was controlled by using a Jeol liquid-N_2 flow system and the temperature was measured with a thermocouple (copper–constantan). For double integrations of e.p.r. spectra, Cu(II)-EDTA was employed as a standard for the spin-quantification of centre S-3 and the free radical signal of *Peptostreptococcus elsdenii* flavodoxin (Massey & Palmer, 1966) was used for ubisemiquinone spin quantification.

E.p.r. spectra were simulated on the University of Pennsylvania Medical School computer facility PDP10. The intensity factor g_p was used to correct for transition probability (Aasa & Vanngaard, 1975).

The e.p.r. spectrum of centre S-3 was simulated by using the spin Hamiltonian $\mathscr{H} = \mu_B S \cdot \hat{g} \cdot H_0$ where μ_B is the Bohr magneton, S the spin, $\hat{g}$ a diagonal second order third rank tensor representing the orientation-dependent spectroscopic splitting factor and H_0 the laboratory magnetic field. A Gaussian lineshape was used: reasonable fits were obtained with a collinear linewidth tensor. The spectrum was obtained as usual by numerical integration over the angles θ and ϕ specifying the possible orientations of the g tensor with respect to H_0.

The dipolar coupled quinone pair spectrum was simulated in the same way, except that the Zeeman spin Hamiltonian $\beta(\vec{S}_1 \cdot \vec{g}_1 \cdot \vec{H}_0 + \vec{S}_2 \cdot \vec{g}_2 \cdot \vec{H}_0)$ was aug-

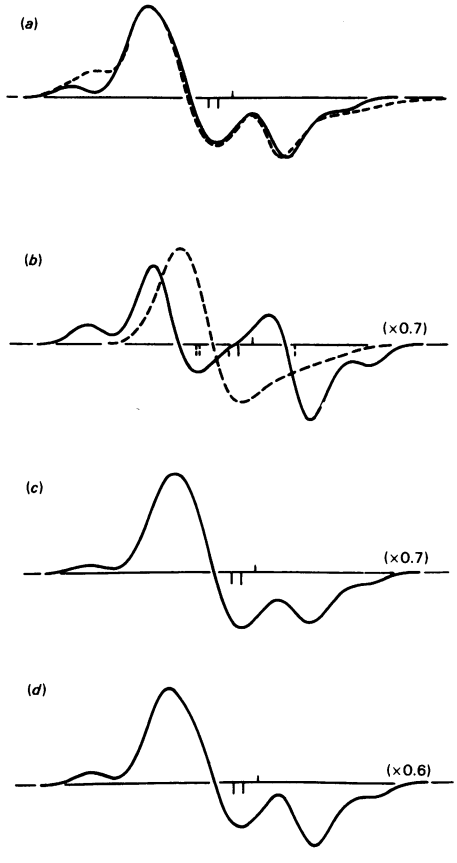


Fig. 1. *Experimentally observed and computer-synthesized e.p.r. spectra of centre S-3 and spin-coupled semiquinone pair*

(*a*) Spectrum of partially-oxidzied beef heart submitochondrial particles (----) and computer-synthesized spectrum (——) with S-3/SQSQ = 0.8:1.0; (*b*) simulated individual spectra of centre S-3 (----) and spin-coupled ubisemiquinone pair (——); (*c*) and (*d*) examples of simulated spectra contributed from both species at various proportions of S-3/SQSQ; (*c*) 1:0.5; (*d*) 1:1. The positive scale marker shows $g = 2.00$. Negative solid scale markers are $g = 2.0066$ and 2.0041; the negative broken scale markers are $g = 2.015$, 2.014 and 1.990, reading from left to right, respectively. Computer simulations were conducted as described in the Materials and methods section. Spectra of centre S-3 and dipolar coupled semiquinone pairs were simulated according to Ruzicka *et al.* (1975); small variations in simulation parameters were introduced to compensate for slight changes in lineshape in different preparations. Parameters used for centre S-3 spectra were $g_x = 1.990$, $g_y = 2.014$, $g_z = 2.015$, $L_x = 2.5 \times 10^{-3}$T (25 gauss), $L_y = 1.3 \times 10^{-3}$T, $L_z = 1.3 \times 10^{-3}$T; for dipolar coupled two-ubisemiquinone spectra, $g_x = 2.0041$, $g_y = 2.0066$, $g_z = 2.0066$, $L_x = 11.5 \times 10^{-4}$T, $L_y = 8 \times 10^{-4}$T, $L_z = 8 \times 10^{-4}$T and $D = 3.0 \times 10^{-3}$T, $\theta_D = 0$, $\phi_D = 3/4 g^2 \mu_B^2 / r^3$; θ_D and ϕ_D specify the orientation of R with respect to the ubisemiquinone g tensors, which are assumed to be parallel since this gave marginally better fits to experimental spectra. $\theta_D = \phi_D = 0$ corresponds to $\vec{R} \parallel \vec{z}$. Comparison of this series with experimental spectra taken at various redox potentials allowed the ratio of semiquinone and centre S-3, and the percentage of maximum of each to be calculated.

mented by a term $g^2 \cdot \beta^2 [r^{-3}(\vec{S}_1 \cdot \vec{S}_2) - 3r^{-5}(\vec{S}_1 \cdot \vec{R})(\vec{S}_2 \cdot \vec{R})]$ where g^2 is the average g values for ubisemiquinone $[(g_x + g_y + g_z)/3]$, r is the effective distance between two ubiquinones with a point dipole approximation, $\vec{S}_1$ and $\vec{S}_2$ are the ubiquinone spins and $\vec{R}$ is the vector connecting the point dipoles. The Hamiltonian was truncated, retaining only terms in S_{1z}, S_{2z}, $S_1^- S_2^+$ and $S_1^+ S_2^-$, where S_{1z} and S_{2z} are the projection of $\vec{S}_1$ and $\vec{S}_2$ along the laboratory field direction and S_n^+ and S_n^- the raising and lowering operators. These simplifying assumptions have no detectable effect under the conditions of small g-value anisotropy and weak coupling $(g\mu_B / r^3 \ll H_0)$. The problem was thus reduced to diagonalization of a 2×2 segment of the 4×4 Hamiltonian matrix at each value of θ and ϕ. The simulations are fairly insensitive to the orientations of the two quinone g tensors with respect to each other and to $\vec{R}$.

Results

In order to resolve the overlapped signals of centre S-3 and the spin-coupled ubisemiquinone pair, contributing in different proportions, the spectrum of partially oxidized submitochondrial particles (broken line) was simulated (solid line) as represented in Fig. 1(*a*). The computer-synthesized spectrum of iron–sulphur centre S-3 (Fig. 1*b*, broken line), as described by Ruzicka *et al.* (1975), was added to the synthesized spectrum of a dipolar-coupled semiquinone pair (Fig. 1*b*, solid line) in the ratio of 0.8:1.0. The deviation from the experimental spectrum at high field is also seen in simulations of centre S-3 alone and may be due to small amounts of modified centre S-3. The deviation seen at lower field is believed to represent the g_z component of an iron–sulphur centre, possibly the Rieske iron–sulphur centre, because the extra peak is not present in Complex II (particulate succinate–ubiquinone reductase) (Ruzicka *et al.*, 1975). Figs. 1(*c*) and 1(*d*) show the simulated lineshape produced by changing the ratio of the two components to 1.0:0.5 and 1.0:1.0. By measuring the ratio of the amplitudes of the two central peaks in experimental spectra and comparing with simu-

lations, the contributions of each component can be determined.

Resolution of the e.p.r. spectra into separately measurable components allows more accurate analysis of titration data. The results of potentiometric titration of the semiquinone pair and centre S-3 in beef heart sub-mitochondrial particles are shown in Fig. 2. It is apparent from the spectra in Fig. 2 that the other features attributed to the ubisemiquinone pair appear and disappear with the line which appears near $g = 1.98$ at x-band. The larger amplitude and better resolution of this feature make it the most accurate measure of the concentration of coupled ubisemiquinones. It would remain a fairly accurate standard even in the event of inhomogeneity in the dipolar coupling strength, which would preferentially broaden the outer pair of lines.

Small changes in the simulation parameters are necessary to fit the spectra of centre S-3 in different preparations (various preparations of submitochondrial particles, succinate–cytochrome *c* reductase and Complex II). Principally, the *g* values vary by approx. 0.002 and the linewidths by a maximum of 5×10^{-4}T. Typical values are given in the Figure legends.

At high potentials, the e.p.r. spectra of submitochondrial particles are characteristic of iron–sulphur centre S-3 (Fig. 2, spectrum *c*). As the potential is lowered the spectral contribution of centre S-3 decreases, whereas the spectral contribution of the Q pair appears and increases (see Fig.

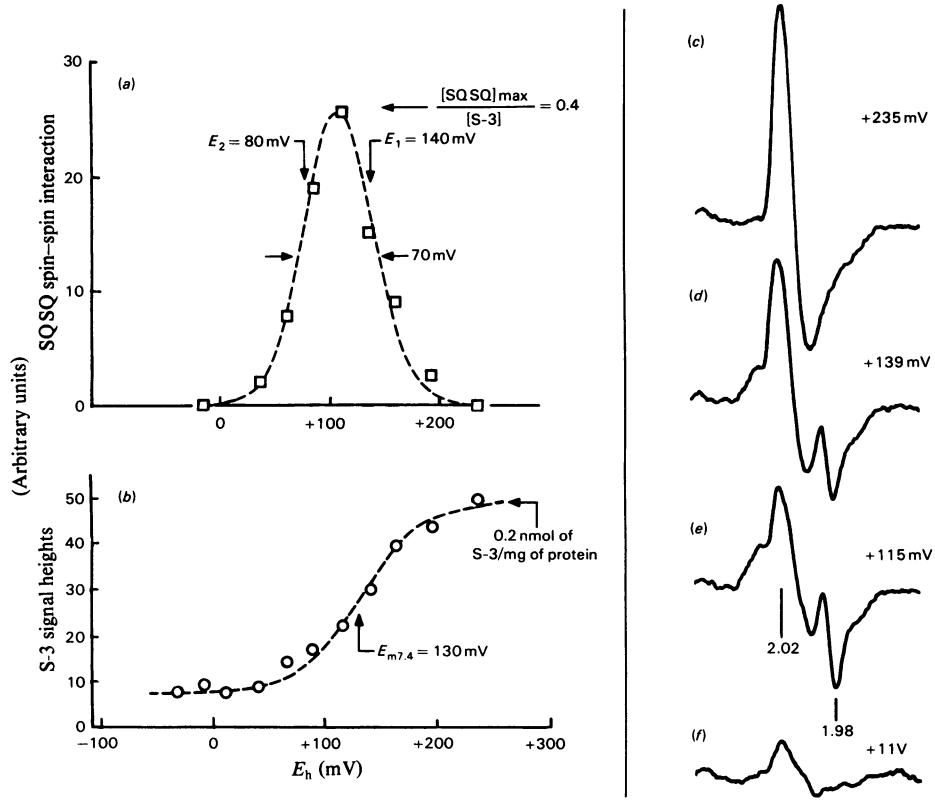


Fig. 2. *Potentiometric titration of e.p.r. signals from dipolar-coupled ubisemiquinone pair and centre S-3*
Submitochondrial particles (18.5 mg of protein/ml) were prepared by sonication of mitochondria in 0.25 M-sucrose/0.1 mM-EDTA/50 mM-Hepes, pH 7.4. Potentiometric titrations were performed as described in the Materials and methods section in the presence of diaminodurene, phenazine methosulphate, 1,4-naphthoquinone disulphonate, pyocyanine, indigo tetrasulphonate, duroquinone, 1,4-naphthoquinone, and 2-hydroxy-1,4-naphthoquinone (the last three were dissolved in dimethyl sulphoxide) at concentrations between 20 and 50 μM. E.p.r. conditions were: microwave frequency, 9.18 GHz; modulation frequency, 100 KHz; modulation amplitude, 8×10^{-4}T; microwave power, 1 mW; time constant, 0.3 s; scan rate, 2.5×10^{-2}T/min; sample temperature, 12 K. Individual spectra (with *g*-values) are illustrated in (*c*)–(*f*).

2, spectra *d* and *b*). Further lowering of the potential causes loss of both signals (Fig. 2, spectrum *f*).

The titration of the signal of the semiquinone pair produces a narrow bell-shaped curve with a peak at 110mV and a full width at half height of 70mV at pH7.4 (Fig. 2*a*). The e.p.r. signal of centre S-3 titrates as a single $n = 1$ component (Fig. 2*b*) with a midpoint potential of 130mV.

Previously we pointed out that the sharpness of the titration curve was to be expected if two ubisemiquinones interacted to produce the observed signal (Ingledew *et al.*, 1976). If each quinone functions as an independent electron (or hydrogen) carrier, the concentration of semiquinone–semiquinone would be proportional to the square of the concentration of individual semiquinone. Fig. 3 shows the possible redox states of a two-quinone system. The equations illustrate the analysis of a particularly simple case, that of two electrochemically equivalent quinones. In these Nernst equations, exp. $a = 10^{(E_h - E_1)/60}$ and exp. $b = 10^{(E_h - E_2)/60}$. The theoretical dependence of the concentration of semiquinone (QH· or $Q^{\bar{\cdot}}$) and spin-coupled semiquinone pair (QH·QH· or $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$) on potential is illustrated in Fig. 4. The closest fit to the data in Fig. 2 is obtained when $E_1 - E_2 = 60$mV. Since $E_{m7.4}$† = 110mV, we conclude that, at pH7.4, $E_1 = 140$mV and $E_2 = 80$mV.

Fig. 5 shows the results of a potentiometric titration of centre S-3 and the e.p.r. signal of the dipole-coupled semiquinone pair at pH7.6 and pH6.6. Except for a small decrease in the maximum intensity attained, centre S-3 is evidently pH-independent in this range. The semiquinone pair signal, on the other hand, is markedly pH-dependent in terms of both maximum intensity and E_m.

The dependence of the maximum intensity of the $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$ e.p.r. signal on pH conveys information on the protonation state of the semiquinone and fully reduced quinone species. The system can be easily analysed by combining Henderson–Hasselbach equations of the form $AH = A^- 10^{(pK - pH)/60}$ with the appropriate Nernst equations. Fig. 6 shows the dependence of the square root of the e.p.r. signal intensity on pH; the square root was taken because in the absence of cooperativity the semiquinone concentration is proportional to the square root of the intensity of the signal of the spin-coupled ubisemiquinone pair.

It is immediately clear that the semiquinone is predominantly in the anionic form ($Q^{\bar{\cdot}}$) over the pH range of the experiment. Otherwise, since the Q/QH· and QH·/QH_2 couples have the same 60mV/pH unit dependence, no stabilization of the semiquinone would be observed at alkaline pH. In fact, an approx. 4-fold stabilization is observed between pH6.5 and pH8.0. Inclusion of a pK_S at 6.2 slightly improves the fit of the model to the data, but this effect is too subtle to conclude with certainty

† $E_{m7.4}$ is the midpoint oxidation–reduction potential determined at a given pH. In this system, E_m is defined as the redox potential at which the peak of the bell-shaped titration curve of the ubisemiquinone is obtained, which is the average value of E_1 and E_2.

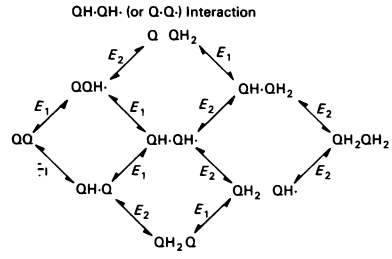


Fig. 3. *Possible redox states of a two-quinone system having equivalent redox properties*

Concentration-dependence of (QH·QH· or $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$) on the midpoint potentials of the first and second electron transfer steps (E_1 and E_2). Exp. $a = 10^{(E_1 - E_h)/60}$; exp. $b \equiv 10^{(E_2 - E_h)/60}$; N, total Q-pair concentration; E_h, oxidation–reduction potential relative to that of hydrogen electrode.

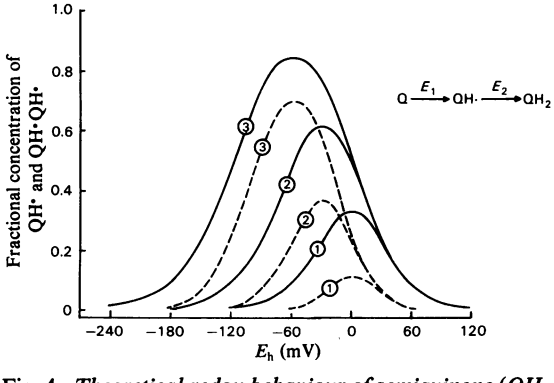


Fig. 4. *Theoretical redox behaviour of semiquinone (QH· or $Q^{\bar{\cdot}}$) and semiquinone–semiquinone (QH·QH· or $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$) species*

——, the individual semiquinone signals; – – –, signals from spin-coupling of the semiquinone pair. 1, $E_2 = 0$; 2, $E_2 = -60$mV; 3, $E_2 = -120$mV, $E_1 = 0$mV.

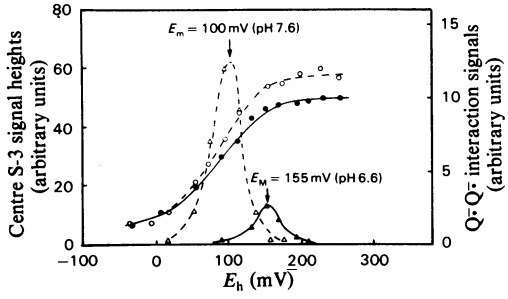


Fig. 5. *Potentiometric titration of e.p.r. signals of centre S-3 and spin-coupled ubisemiquinone pairs at pH6.6 and 7.6*

Submitochondrial particles (22.0mg of protein/ml) were suspended in 0.25M-mannitol/0.1mM-EDTA/50mM-Mops (for pH6.6) or Hepes (for pH7.6). Potentiometric titration was performed as described in the legend to Fig. 2. 1,4 Naphthoquinone disulphonate, pyocyanine, indigo tetrasulphonate, indigo disulphonate, duroquinone, 1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone and *NNN'N'*-tetramethyl-*p*-phenylenediamine (the last four dissolved in dimethyl sulphoxide) were added at the final concentration of 50μM. ——, Titrations at pH6.6; – – – –, at pH7.6, ○, ●, Signals from centre S-3; △, ▲, signals from spin–spin interactions of ubisemiquinone pairs.

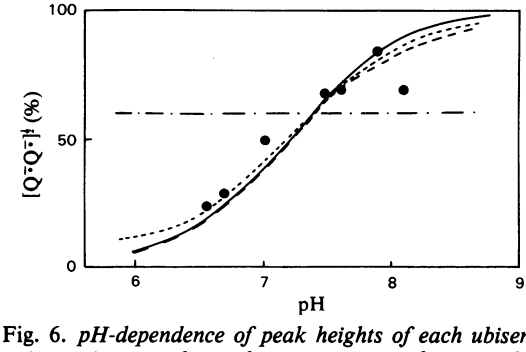


Fig. 6. *pH-dependence of peak heights of each ubisemiquinone titration obtained as a square root function of the $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$ interaction signals relative to the total S-3 content*

Theoretical curves: ——, $pK_S \ll 6$; ····, $pK_S = 6.2$; – – –, $pK_R = 7.9$; —·—·—·, both QH· and QH_2 are in the protonated form in the whole pH range tested.

that the pK_S is near 6.2. It is, however, possible to rule out values of pK_S greater than approx. 6.5. More convincingly, in Fig. 5 it can be seen that the high-potential tail of the titration curve of the dipolar coupled species is slightly (approx. 20mV) shifted between pH6.6 and pH7.6. This effect can only be reproduced mathematically by a value of pK_S slightly below 6.5.

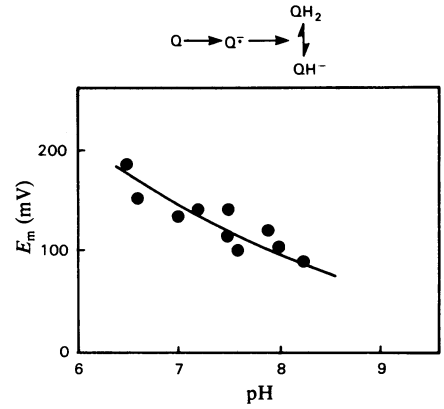


Fig. 7. *Midpoints of the potentiometric titrations from spin-coupled ubisemiquinone ($Q^{\bar{\cdot}}Q^{\bar{\cdot}}$) as a function of pH*

The theoretical line assumes a pK_R of 7.9.

The pH-dependence is in general less sensitive to pK_R than to pK_S; assuming $pK_R \neq pK_S$, at pK_R the dependence of $(E_1 - E_2)$ on pH merely changes from 120mV/pH unit to 60mV/pH unit, whereas at pK_S this dependence changes from 0mV/pH unit to 120mV/pH unit. We can still rule out a value of pK_R below 7.5, but a pK_R of about 8.0 is consistent with the data. The pH dependence of the $E_m[(E_1 + E_2)/2]$ value for the $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$ signal depends only on the value of pK_R. Above pK_R, the dependence of E_m on pH is 30mV/pH unit; below pK_R, a 60mV/pH unit dependence is expected. The data are summarized in Fig. 7. The typical scatter inherent in the titration technique and the rather narrow pH range available make detailed conclusions unconvincing, but the best fit lies between the above two extremes. If a pK_R between 7.5 and 8.0 is included (solid line), a somewhat better fit can be obtained.

The model introduced in Fig. 3 assumed no co-operativity. However, in the $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$ state, Coulomb interactions are potentially important. If the assumption of electrochemically equivalent semiquinones is retained (this leads to the best fits), co-operativity can be introduced by using three terms that define the effects of the redox state of one ubiquinone on the E_m values of the couples of the other. The best fits were obtained when all three parameters were less than 30mV; therefore we do not expect co-operativity to be of functional importance. It could, however, affect the concentration of semiquinone observed.

The magnitude of the Coulomb energy between two unprotonated semiquinones 1nm apart can be estimated by using a point charge approximation as $1.44/\varepsilon$ (volts) $\times$ e, were e is the charge on an electron and ε is the effective dielectric strength. This potentially large Coulomb contribution to the

energy of the ubisemiquinone–ubisemiquinone state could be minimized in two ways. One semiquinone could become protonated at a pH above the pK of the semiquinone in the ubisemiquinone–quinone redox state. If dipolar Coulomb terms are neglected, this unfavourable protonation would itself destabilize the ubisemiquinone–ubisemiquinone state by about 100mV at pH8.0; inclusion of dipolar terms would lessen this somewhat. The observed stabilization of the ubisemiquinone–ubisemiquinone state at alkaline pH argues against this mechanism, however. Alternatively, the Coulomb interaction will be weaker if ε is large. The value of ε for bulk lipids is near 2; ε can range up to about 80 for water, although there is reason for caution in applying bulk values of ε on a molecular scale. These considerations suggest that the semiquinone pair may be in a polar region.

Fig. 8 shows e.p.r. spectra of centre S-3; the spin–spin interaction signals of the $Q^{\bullet-}Q^{\bullet-}$ pair are completely quenched in the presence of 100μM-TTFA. It is also obvious that centre S-3 titrates at lower potential in the presence of TTFA (Figs. 8a' and 8b').

These effects are reflected in Fig. 9, which gives the results of the titration in the presence and absence of TTFA. Whereas the bell-shaped titration curve of the spin-coupled $Q^{\bullet-}Q^{\bullet-}$ signal is completely quenched, the sigmoidal $n=1$ titration curve of centre S-3 does not change in shape or intensity; however, its midpoint redox potential shifts from +130 to +45mV.

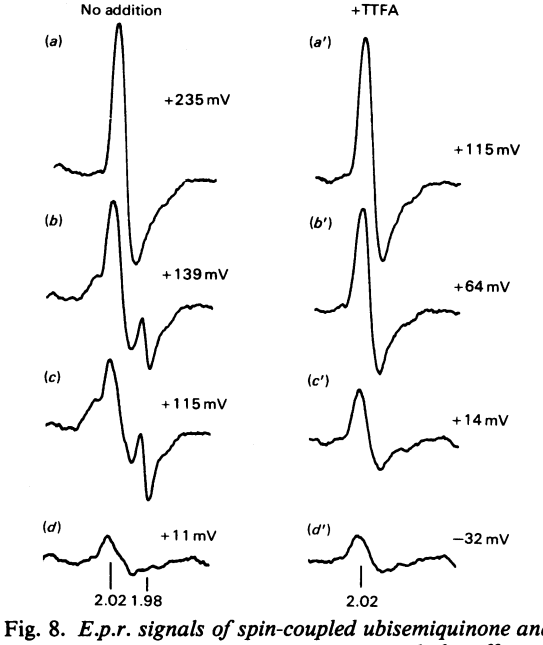


Fig. 8. *E.p.r. signals of spin-coupled ubisemiquinone and centre S-3 in potentiometric titration and the effect of TTFA on these parameters*

Potentiometric titrations were performed as described in Fig. 2; the concentration of TTFA was 100μM. E.p.r. conditions were as described in Fig. 2.

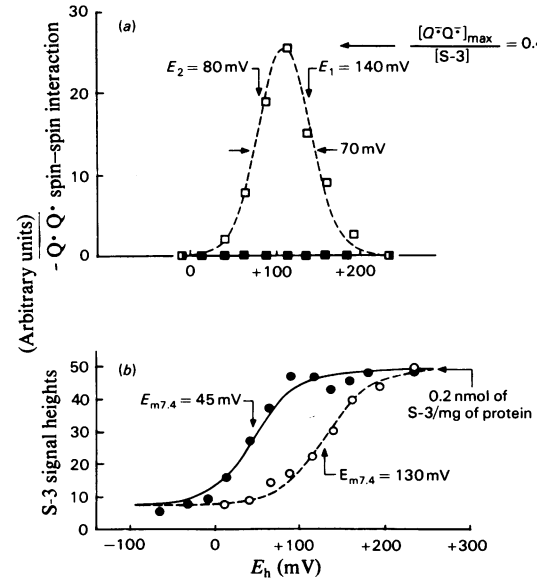


Fig. 9. *Potentiometric titrations of ubisemiquinone–ubiquinone spin–spin interaction (a) and centre S-3(b) in the presence and absence of 100μM-TTFA*

TTFA at the final concentration of 100μM was dissolved in dimethyl sulphoxide and preincubated with sub-mitochondrial particles for 5min before conducting the potentiometric titration. Other experimental conditions are the same as in Fig. 2. Open symbols and ----, control; solid symbols and ——, +TTFA.

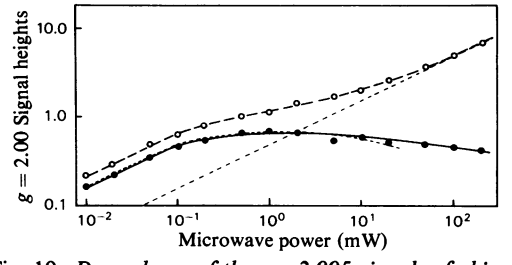


Fig. 10. *Dependence of the g = 2.005 signals of ubisemiquinone on the input microwave power in the presence (●——●) and absence (○——○) of added TTFA*

The control sample was poised at $E_h = 95$mV and the TTFA-treated sample at $E_h = 73$mV. The biphasic saturation curve obtained in the absence of TTFA was resolved into two component curves as indicated by (·····). E.p.r. signals were monitored at a sample temperature of 203K.

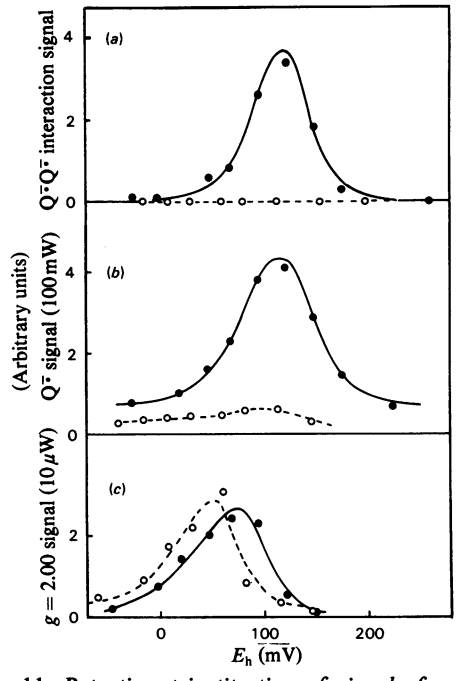


Fig. 11. *Potentiometric titration of signals from spin-coupled ubisemiquinone pair and the g = 2.005 signals of two different species of ubisemiquinone in the presence* (●——●) *and absence* (O----O) *of TTFA*

The $Q^{\cdot}Q^{\cdot}$ interaction signals presented in (*a*) were measured as described in the legend to Fig. 1 and plotted as a function of E_h. The titration curves in (*b*) and (*c*) show the peak-to-peak amplitude of the $g=2.005$ signals measured at 100mW and 10μW input microwave power, respectively, at 207K. The titration was conducted on a beef heart submitochondrial particle suspension at a final protein concentration of 21.8mg/ml in the presence of the following redox dyes: 1,4-naphthoquinone, 1,2-naphthoquinone, 1,4-naphthoquinone disulphonate, duroquinone, indigo disulphonate and 2-hydroxy-1,4-naphthoquinone, each at 50μM. The particle suspension was incubated with 100μM-TTFA for 10min at room temperature before conducting the titration.

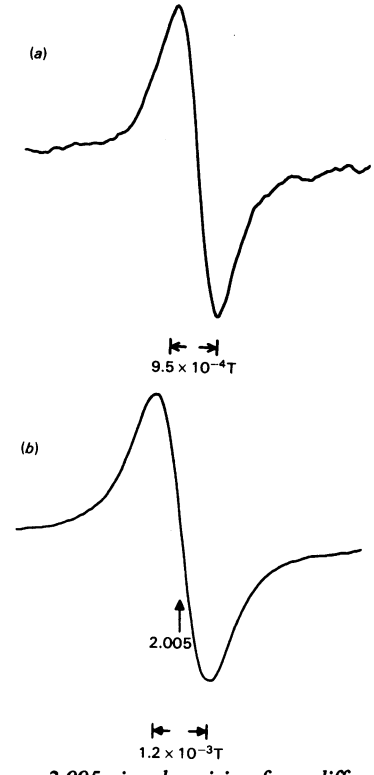


Fig. 12. *g = 2.005 signals arising from different species of ubisemiquinone monitored in a submitochondrial particle suspension*

Spectrum (*a*) (10μW) arises from QH_c and spectrum (*b*) from QH_s; both were recorded at a sample temperature of 203K. The redox potential of sub-mitochondrial particle (pH 7.4, 40mg of protein/ml) was potentiometrically poised to 77mV (*a*) or 115mV (*b*) in the presence of redox-mediating dyes as described in Fig. 11. The level of microwave input power was as shown illustrated in the Figure; modulation amplitude, 8×10^{-4}T.

The e.p.r. spectra recorded at higher temperature (203K) show unsplit signals near $g = 2.005$. These signals arise from redox states such as $QQ^{\cdot}$ or $QH_2Q^{\cdot}$ from the same ubiquinone pool in which the $Q^{\cdot}Q^{\cdot}$ state gives rise to spin-coupled split signals observed at much lower temperatures. In beef heart sub-mitochondrial particles poised at +110mV, the power-saturation characteristics of the free radical signals are biphasic and can be resolved into two components, as shown in Fig. 10. One component saturates with $P_{\frac{1}{2}}$ of approx. 0.4mW, whereas the other is unsaturated even at 100mW. These two free radical species, having different relaxation behaviour, can be selectively monitored at two extreme microwave power levels, namely 10μW and 100mW. At these two power settings, the interfering contribution from the other component is about 25 and 10%, respectively. Addition of 100μM-TTFA completely quenches the more rapidly relaxing species, but does not affect the slowly relaxing species.

Fig. 11 represents potentiometric titrations of the ubisemiquinone signals from different pools and the $Q^{\cdot}Q^{\cdot}$ interaction signals. The $Q^{\cdot}Q^{\cdot}$ signal and the $g = 2.00$ signal monitored at a high microwave power (100mW) show the same E_m values (120mV) and are concomitantly quenched by 100μM-TTFA. The rapidly relaxing $g = 2.00$ signals give a bell-shaped titration curve with a wider full width at half-height (about 90mV) than that of the $Q^{\cdot}Q^{\cdot}$

titration (about 60mV) as discussed earlier (see Fig. 4). The slowly relaxing $g = 2.00$ signals monitored at 10μW input power give a somewhat skewed titration curve that reaches a peak at lower redox potential (80mV). This ubisemiquinone species gives a maximal free-radical concentration in the range of 5–15% of that of the cytochrome c_1 present in the submitochondrial particle suspension (0.8nmol/mg of protein). Under these conditions we calculate $E_1 = 20$–40mV, $E_2 = 120$–140mV. Addition of TTFA (100μM final concentration) has a minor effect on the slowly relaxing ubisemiquinone species; it causes an apparent negative shift in the peak position by 20mV.

The e.p.r. spectra of the $g = 2.00$ signals arising from the two different ubisemiquinone species observed in the mitochondrial membrane are presented in Fig. 12. The overlapped spectra were resolved by applying two extremely different levels of input microwave power. Spectrum (*a*) is attributable to SQ_c and has a peak-to-peak width of 9.5×10^{-4}T. In contrast, SQ_s, spectrum (*b*) monitored at higher microwave power, shows a wider (1.2×10^{-3}T) peak-to-peak width. The spectrum of SQ_c exhibits a Gaussian line shape, whereas that of SQ_s has a more Lorentzian shape.

Discussion

The most striking feature of the results of the potentiometric titrations is the high stability of the ubisemiquinone radicals. (For the purposes of this discussion, we use the term stability in the thermodynamic sense.) The stability constant of free ubisemiquinone in a hydrophobic milieu has been estimated to be 10^{-10} (Mitchell, 1976) at pH 7.0; the stability constants of the quinones in the dipolar coupled pair, which were estimated from the spin-coupled signals, are greater than one (K approx. 10) whereas that of the 'slowly' relaxing quinone was determined as approx. 5×10^{-2}, assuming that the pool size of this Q species is equivalent to the cytochrome c_1 concentration. We distinguish these stabilized ubisemiquinone species as SQ_s and SQ_c, because the former appears to arise from the Q species in the succinate–ubiquinone reductase segment and the latter from that in the ubiquinol–cytochrome *c* reductase segment of the respiratory chain (Ohnishi & Trumpower, 1980).

The shared sensitivity to TTFA and the similar midpoint potentials (E_m) of the spin-coupled $Q^{\cdot-}Q^{\cdot-}$ and $g = 2.00$ radical species measured at 100mW at 207K suggest that they arise from the same pool of ubisemiquinone, namely SQ_s. The presence of the highly stabilized intermediate redox state and the previously reported fixed orientation in the membrane of the Q pair (Ohnishi *et al.*, 1977; Salerno *et al.*, 1979) suggest that the quinones are bound to a membrane protein. This may account for the rather unusual electrochemistry of these Q species, since preferred binding of $Q^{\cdot-}$ might stabilize the ubisemiquinone species with respect to the quinone and quinol forms. This requires that the semiquinone forms be bound at least several orders of magnitudes more tightly than the ubiquinone and ubiquinol forms. The pK values might be similarly controlled by binding to a protein site.

If the bound quinone molecules dissociate on a physiologically significant timescale, it is unlikely that they do so as semiquinones. The quinone and quinol forms, much less tightly bound, might dissociate and diffuse. We consider it more likely that the quinone pair at least remain bound to a protein on a catalytic timescale; they may function as reductants for other mobile Q molecules.

The presence of binding sites that stabilize the ubisemiquinone is not surprising; it is useful and perhaps necesary in view of the overall structure of the electron transfer system in mitochondria. Obligatory two-electron reductants such as NADH and succinate reduce flavins in a two-electron step. In succinate dehydrogenase, the flavosemiquinone is stable enough to permit the reduction of the iron–sulphur centres of the dehydrogenase in sequential one-electron steps; both the upper and lower couples of the FAD in succinate dehydrogenase are lower in potential than two of the iron–sulphur centres. A binding site that stabilizes ubisemiquinone will allow the reduction of Q by iron–sulphur centres in sequential steps by one- or two-electron oxidant/reductant. The Q-binding proteins, QP_s and QP_c, proposed by Yu *et al.* (1977), are attractive candidates. Cytochrome *b* or Rieske's iron–sulphur protein could also be binding sites.

Without such binding sites, the dehydrogenases would need to be able to transfer two electrons to Q simultaneously, since the first one-electron reduction would be very unfavourable. This is probably an important factor in the inability of all solubilized succinate dehydrogenases to reduce free Q, and it is likely that similar sites that stabilize ubisemiquinone permit the entry of single electrons from other dehydrogenase branches.

The slowly relaxing, TTFA-insensitive, semiquinone (SQ_c), although greatly stabilized compared with free Q, still has a K of considerably less than 1. This means that the second electron removed from the ubiquinol is more reducing than the first. The $QH_2/Q^{\cdot-}$ couple for a quinone bound at this site has an $E_{m7.4}$ of +120–+140mV, making it a good reductant for cytochrome c_1 or Rieske's centre but a poor reductant for the *b* cytochromes. The $Q^{\cdot-}/Q$ couple has an $E_{m7.4}$ of +20–+40mV, making it a feasible reductant for the *b* cytochromes. The purpose of a binding site that only partially stabilizes

the semiquinone might be to split the electron pair, one electron going from quinol through the Rieske iron–sulphur centre and cytochrome c_1 to oxidase and another back through an electron-transferring arm containing the *b* cytochromes. The modest residual instability ($E_1 - E_2$ approx. −90mV) enables the reduction of lower-potential components to be driven by cytochrome c_1 reduction.

Both the nearly isotropic $g = 2.00$ type signal and the split signals of the dipolar coupled pair have been observed in green-plant mitochondria from several species (Rich *et al.*, 1977) and in mitochondria from *Neurospora crassa* (Rich & Bonner, 1978). Unfortunately, the split signal is eradicated by small concentrations of organic solvent, although the mitochondria remain active; this has prevented the use of potentiometric techniques on these systems. Quinone radicals have not been observed in membranous preparations from photosynthetic bacteria (Takamiya & Dutton, 1979), which contain a succinate dehydrogenase apparently similar to the mammalian enzyme.

Since the split signal is caused by the interaction of two paramagnetic molecules, it is not surprising that it would be much more sensitive to perturbing influences than signals arising from a single group. A modest shift (40mV) in the midpoint potential of one quinone relative to the other would cause almost complete loss of signal. Even if the quinones remained equivalent, modest decreases in the binding constants of the semiquinones would cause a large decrease in the observed $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$ signal, since it depends on the square of the $Q^{\bar{\cdot}}$ concentration.

In addition, introduction of small amounts of inhomogeneity in the positions of the dipolar coupled quinones would cause lineshape changes. When the major contribution to the linewidth is due to this type of inhomogeneity, the four-line spectrum collapses, first to a pseudo doublet with very weak wings and then, as inhomogeneity further increases, to a broad, roughly isotropic, line. During this process, the signal amplitude sharply declines. It is interesting that Rich *et al.* (1977) observed broad free radical signals consistent with these mechanisms in plant mitochondria when the dipolar coupled signal was lost through addition of organic solvents or excessive sonication.

The rapid low-temperature relaxation of the quinone pair is more characteristic of transition metals than of organic free radicals. Interactions with nearby electron carriers, especially iron–sulphur centre S-3, could provide a cross-relaxation mechanism. While $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$ interaction might account at least in part for the rapid relaxation of the quinone pair, the enhanced relaxation of the unsplit quinone radical (Fig. 10) visible at higher temperature must have other causes. As we previously suggested, interaction with transition-metal-containing groups is the simplest explanation. Since TTFA blocks electron flow between centre S-3 and Q, the e.p.r. signal from the $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$ pair is specifically quenched by TTFA and the midpoint potential of centre S-3 is shifted, centre S-3 is the most attractive candidate for the other partner in the interaction. Reduced centre S-3 is probably not diamagnetic; although it does not give rise to e.p.r. signals, only the ground state has zero spin ($S = 0$). The low-lying excited states, with $S = 1$, 2 etc., may provide potent cross-relaxation mechanism.

The existence of an ordered Q pair oriented to span a significant fraction of the mitochondrial membrane and functioning as an electron acceptor from succinate dehydrogenase suggests its function in proton translocation. Although previous interest centred around Q as a mobile hydrogen carrier (Kröger & Klingenberg, 1973; Mitchell, 1976), and evidence has recently been presented that purports to link these Q pools to the Q cycle (Konstanchinov & Ruuge, 1977*b*), we prefer a more 'solid-state' interpretation of the results.

If bound Q assemblies penetrate the membrane, Site-II proton translocation might be mediated by the passing of protons from quinol to quinone, possibly mediated by bound water molecules. The need for transmembrane movement of Q would thus be obviated, although lateral movement of free (diffusible) quinone might still mediate between electron carriers on the same side of the membrane (Heron *et al.*, 1978; Schneider *et al.*, 1979). Models closely analogous to the Q cycle can be constructed in this way, and both the $2H^+/e^-$ ratio and oxidant-induced cytochrome *b* reduction can be retained as illustrated schematically in Scheme I.

Previously, we reported that antimycin caused increased concentrations of ($Q^{\bar{\cdot}}Q^{\bar{\cdot}}$) and ($Q^{\bar{\cdot}}$) of the SQ_s pool in mitochondria and sub-mitochondrial particles in the presence of fumarate and ascorbate or succinate and ferricyanide (Salerno *et al.*, 1978). As a possible explanation, we pointed out that, if antimycin inhibited the uptake of H^+ by the $Q^{\bar{\cdot}}$ pair [as in the case of H^+_{II} in the bacterial chromatophore system (Codgell *et al.*, 1972; Petty *et al.*, 1979)]. $Q^{\bar{\cdot}}$ would be stabilized relative to QH_2. Direct potentiometric titration of the ubisemiquinone $g = 2.00$ signal of the ubisemiquinone species SC_c and SQ_s and signals from the spin-coupled $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$ pair conducted more recently with the succinate–cytochrome *c* reductase complex (Ohnishi & Trumpower, 1980), has shown that antimycin A stabilizes SQ_s relative to its oxidized form (Q) rather than to its fully reduced form (QH_2); concomitantly antimycin A completely destabilizes the SQ_c signal. Thus antimycin influences the binding of quinone at the Q_s site; either Q is less tightly bound in the presence of antimycin, or $Q^{\bar{\cdot}}$ and QH_2 are more tightly bound.

As we have mentioned, it is possible that these

bound quinones are part of a solid-state mechanism for proton translocation. The intermediate redox state of the quinones in the pair is $Q^{\bar{\cdot}}$; the couple $Q^{\bar{\cdot}}QH_2$ inherently has a $2H^+/e^-$ ratio. Any electron-carrying arm (presumably utilizing the *b* cytochromes) therefore need not span the entire membrane in order to preserve the $2H^+/e^-$ ratio. The *b* haems need not be on opposite sides of the membrane to explain the ATP-induced shift in the relative midpoint potentials if H^+ is delivered to one *b* cytochrome through a proton well, perhaps even the hydrophilic $Q^{\bar{\cdot}}Q^{\bar{\cdot}}$ binding pocket; the effect of $\Delta\psi$ would in this case be on the motion of H^+, not e^-. That such an arm does exist, at least in foreshortened form, is implied by oxidant-induced reduction of the *b* cytochromes. The simplest explanation for this phenomenon is the oxidation of a quinol (with an unstable semiquinone state) to the quinone by a *b* cytochrome and c_1 [possibly with the Rieske centre as an intermediate (Trumpower *et al.*, 1979)]. This sort of explanation, first proposed by Wikström & Berden (1972), implies a non-linear electron transport chain; as pointed out by Mitchell, the best rationale for such a non-linear chain is an electron-transferring arm such as that proposed in the original Q cycle (Mitchell, 1976). The notion that a large part of the proton-translocating mechanism is 'solid state' is strengthened by the observed actions of antimycin. The multiple effects of the binding of a single (or at most two) molecule(s) of antimycin per chain are more easily rationalized if the components affected [*b* cytochromes (Pumphrey, 1962) and the Q pair associated with succinate dehydrogenase and SQ_c (Salerno *et al.*, 1978; Ohnishi & Trumpower, 1980)], are all closely associated parts of a larger unit (Complex II and III). This is entirely in keeping with the recent kinetic studies on Complexes I and III, in which electron transfer (NADH–cytochrome *c* reductase) was found to occur maximally through 1:1 complexes of Complexes I and III (Heron *et al.*, 1978).

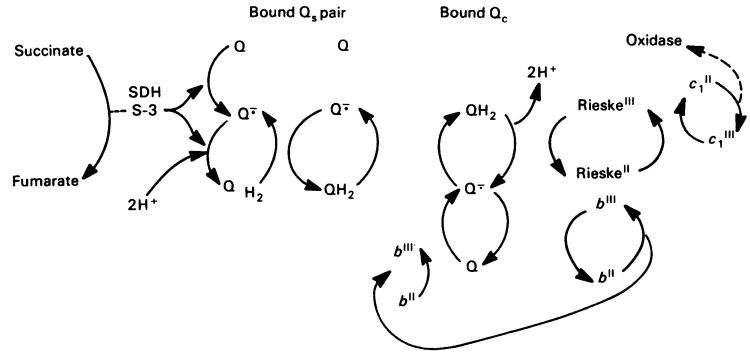
The actions of TTFA on the Q pair are more specific, but it is not yet clear whether TTFA replaces the quinones or merely affects their electrochemistry, either thermodynamically (again by modification of binding constants) or kinetically (possibly by steric effects). The proposal of Trumpower & Simmons (1979), that TTFA specifically inhibits the reduction of semiquinone to quinol by succinate dehydrogenase but does not affect the reduction of quinone to semiquinone, is in direct contradiction with the experimental finding that TTFA causes quenching of the semiquinone in potentiometric titrations (Figs. 9 and 11).

If TTFA inhibited only the reduction of $Q_s^{\bar{\cdot}}$ to QH_2 by succinate dehydrogenase, $Q_s^{\bar{\cdot}}$ should accumulate under reducing conditions. If other pathways of $Q_s^{\bar{\cdot}}$ reduction (dismutation) were fast enough to prevent a significant increase in $Q_s^{\bar{\cdot}}$ concentration, no inhibition would be observed since the rate-limiting step ($Q_s^{\bar{\cdot}}$ to QH_2 by definition) would be bypassed.

In fact, the radical signals are quenched by TTFA. The conclusions of Trumpower & Simmons are based on the $O_2^{\bar{\cdot}}$-dependent reduction of cytochrome *c* in the presence of antimycin, which is, at least under some conditions, stimulated by TTFA. It is interesting to note that TTFA and antimycin have opposite effects on the stability of $Q_s^{\bar{\cdot}}$. In the presence of antimycin the $Q_s/Q_s^{\bar{\cdot}}$ couple has an $E_{m7.2}$ of nearly 200mV; it is conceivable that this is too high to produce efficiently superoxide anion. Addition of TTFA destabilizes $Q_s^{\bar{\cdot}}$, possibly by greatly lowering the potential of the $Q_s/Q_s^{\bar{\cdot}}$ couple (it could also raise the midpoint of the $Q_s^{\bar{\cdot}}/QH_2$ couple). Even if, as postulated by Trumpower & Simmons, $Q_s^{\bar{\cdot}}$ is the reductant that produces the $O_2^{\bar{\cdot}}$, the synergistic effect of antimycin and TTFA on $O_2^{\bar{\cdot}}$ production might simply be a result of balancing the effects of the two inhibitors so that the $Q_s/Q_s^{\bar{\cdot}}$ couple is low enough in potential to reduce O_2 to $O_2^{\bar{\cdot}}$, yet high enough to be reducible by succinate. TTFA could also act to increase the accessibility of the site to O_2. In any case, the findings of Trumpower & Simmons certainly do not demonstrate the differential inhibition of the reduction of $Q^{\bar{\cdot}}$ to QH_2 by succinate; no further series of similar experiments using only the rate of reduction of cytochromes *c*, *b* or bulk quinone by succinate is likely to prove any more useful. In fact, the inhibition by TTFA of the reduction of bulk quinone by Q_sH_2 has not yet been rigorously excluded. Further meaningful studies in this area must include a direct method of determining the redox state of Q_s, which is easily accomplished by e.p.r.

Konstanchinov & Ruuge (1977*b*) reported that TTFA also quenches slowly-relaxing Q. It should be pointed out that these investigators used a system equilibrated with succinate/fumarate couple, and TTFA at an extremely high concentration (5mM). We noticed that even 250μM-TTFA exhibits a nonspecific destabilization effect on QH_c, namely, about 25% diminished maximal QH_c signal in the potentiometric titration (results not shown).

The Q-cycle scheme put forward by Konstanchinov & Ruuge (1977*b*) to explain the observation of diminished amounts of semiquinone in the presence of succinate and ferricyanide fails to account for the formation of radical in the presence of fumarate and ascorbate (Salerno *et al.*, 1978). In addition, the redox behaviour of the quinones that apparently function as the electron acceptors from succinate dehydrogenase is such that there is no reason *a priori* why succinate dehydrogenase should not reduce both Q to $Q^{\bar{\cdot}}$ and to QH_2. The *b* cytochromes are better reductants than the $Q^{\bar{\cdot}}/Q$



Scheme 1. *Tentative scheme of H^+ and e^- transfer in the Site-II segment of the respiratory chain*
SDH, succinate dehydrogenase; *b*, *c*, cytochromes *b* and *c*.

couple of the bound Q pair. Thus the formation of QH_2 from quinone (other than the bound pair) with a QH_2 of the bound pair and cytochrome *b* as reductants could be easily rationalized without artifical restrictions on the interaction of Q with various couples. The availability of protons in the hydrophobic regions of the membrane might serve (along with redox potential and steric effects) to control the reactions of the various quinone couples. For example, this could be used to ensure that Q_sH_2 and not Q_sH^- served as the reductant for additional quinone molecules, preserving a high H^+/e^- ratio at slightly alkaline pH.

Scheme 1 is capable of accounting for the observation discussed here. We do not know if other quinones (either bound or diffusible) participate between Q_s and Q_c. It is also not clear whether cytochromes *b* reduce Q_c, or an intermediate quinone, or a diffusible quinone that will be bound at the Q_c site for oxidation, or whether the electron-transferring arm is somewhat longer. In the last case, Q_sH_2 might supply both electrons that eventually appear at Q_c; the *b* cytochromes would then reduce the Q_s thus formed to $Q_s^{\bar{}}$. In contrast, in Scheme 1 the $Q_s^{\bar{}}/Q_sH_2$ supplies one electron and two protons for the reduction of Q to QH_2 (the second electron is supplied by a *b* cytochrome) at one site, whereas at another site QH_2 is oxidized to Q by cytochromes *b* and c_1 (through Rieske's iron–sulphur centre).

As more information about the respiratory chain is uncovered, these models will almost certainly have to be modified or discarded; we present them as a framework for the planning of experiments and the rationalization of their results. Before the true scheme of electron and proton transfer in this complex system can be deduced a great deal must be known, especially about the response of these couples to membrane potentials and/or pH gradients, and the location of components in the mitochondrial membrane.

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