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ON THE ROLE OF UBIQUINONE IN THE RESPIRATORY CHAIN

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(1) The V_1 (substrate-Q oxidoreductase activity) and V_2 (QH_2 oxidase activity) for the oxidation of substrates by submitochondrial particles have been measured by using heptylhydroxyquinoline *N*-oxide (HQNO) as inhibitor of V_2. (2) Partial destruction of the Rieske Fe-S cluster by treatment with BAL (2,3-dimercaptopropanol) + O_2 has the same effect on the QH_2 oxidase activity as partial saturation of the antimycin-binding site with HQNO. (3) The extent of the rapid reduction of cytochrome *b* in the presence of excess antimycin is proportional to the percentage of intact Rieske Fe-S cluster. (4) The measured rate of oxidation of endogenous ubiquinol (V_2) by submitochondrial particles is dependent on the substrate used to reduce ubiquinone, especially at low levels of ubiquinone. (5) Pool-function kinetics in the oxidation of substrate, found both in the presence and absence of free ubiquinone, are due both to the pool of free ubiquinone and to direct collision between Q-loaded Q-reducing and -oxidizing enzymes. At infinite Q content only the former mechanism is operative; at low Q content only the latter. (6) Duroquinone can be reduced directly by NADH dehydrogenase without mediation of ubiquinone, but duroquinol cannot be oxidized in the absence of ubiquinone. On the other hand, the reduction of cytochrome *b* by duroquinol does not require the presence of ubiquinone. (7) It is suggested that the need for ubiquinone for the oxidation of duroquinol is due to the requirement of ubisemiquinone for the oxidation of cytochrome *b*, duroquinol not being able to form a stabilized semiquinone.

Introduction

Interest in the role of ubiquinone as an electron or hydrogen carrier in the respiratory chain has been greatly stimulated by the postulated role of the ubisemiquinone in electron transfer [1–4], the concept of the protonmotive Q-cycle [5] and the evidence for the presence of Q-binding proteins [6]. Several recent reviews [7,8] and a whole meeting [9] have been devoted to the role of ubiquinone. The basic concept is still the idea of Green [10], tested by Kröger and Klingenberg [11] and more recently advocated by Schneider et al. [12], that ubiquinone is a mobile carrier, mediating between the various dehydrogenases and the QH_2-cytochrome *c* reductase. The pool-function kinetics, investigated by Kröger and Klingenberg [11], can be described by the equation $v = V_1 \cdot V_2/(V_1 + V_2)$ where v is the rate of oxidation of substrate by oxygen, V_1 is the rate of reduction of Q when all Q present is oxidized and V_2 is the rate of oxidation of QH_2 when all Q present is in the reduced form. To test this equation Kröger and Klingenberg used antimycin in order to decrease V_2. In this paper we show that the use of antimycin introduces complications, because of its cooperative effects [13], but that with HQNO as inhibitor the formula is consistent with the observed steady-state kinetics and can be used to

Abbreviations: BAL, British Anti-Lewisite (2,3-dimercaptopropanol); HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.

measure V_1 and V_2.

Ragan and co-workers [14,15] have shown that another possible interpretation of the steady-state kinetics is the mobility of the respiratory-chain enzymes themselves, rather than the mobility of ubiquinone. In this paper we show that indeed free mobile ubiquinone is not necessarily required for apparent pool-function behaviour and that both direct interaction between Q-loaded enzymes and diffusion of ubiquinone (and ubiquinol) are involved in the electron transfer from the dehydrogenases to the QH_2:cytochrome *c* oxidoreductase. Furthermore, it is demonstrated that rapid electron transfer from the Fe-S cluster in one respiratory-chain assembly to the antimycin-binding site in another does not take place. Since there is good reason to believe that a ubisemiquinone is involved in this segment of the respiratory chain, it follows that this semiquinone does not have a pool function. This is in agreement with the concept that the ubisemiquinone can only exist when stabilized by binding to a protein.

Finally, it is shown that ubiquinone is required for the oxidation of duroquinol, but not for the reduction of duroquinone by NADH.

Materials and Methods

Submitochondrial particles were prepared from beef-heart mitochondria [16] following the procedures as described in Refs. 17 (ATP-Mg particles) and 18 (EDTA particles).

Substrate-oxidation rates were measured with an Oxygraph, equipped with a Clark-type oxygen electrode.

The rate of reduction of duroquinone by NADH was measured spectrophotometrically, following the decrease of the absorbance of NADH in the presence of antimycin and cyanide at 340 nm.

Reduction of cytochromes was measured with an Aminco-Chance DW-2 Spectrophotometer, equipped with a thermostatically controlled cuvette holder.

Treatment with BAL was carried out by preincubating submitochondrial particles (10 mg/ml) with various amounts of BAL for 40 min at 30°C with shaking. During the preincubation, 1 mM malonate was present to activate succinate dehydrogenase so that full activity of succinate oxidase could be subsequently measured. After incubation, free BAL and free malonate were removed by sedimenting the particles at $200000 \times g$.

Antimycin was obtained from Nutritional Biochemical Corporation and HQNO from Sigma. Both were added in ethanolic solutions such that the final ethanol concentration did not exceed 1% (v/v). The concentrations were determined spectrophotometrically as described in Ref. 19.

Protein was measured using the biuret reaction after precipitation of protein with trichloroacetic acid [20].

Results

The use of antimycin and HQNO to test the pool function behaviour of ubiquinone

The validity of the equations for the pool function of ubiquinone

$$v_1 = V_1 \frac{[Q]}{Q_t}, \quad v_2 = V_2 \frac{[QH_2]}{Q_t},$$

where v_1 and v_2 are the actual rates of reduction and oxidation, respectively, of ubiquinone (ubiquinol), V_1 and V_2 the maximal rates of these activities, when all ubiquinone (Q) is in the oxidized or the reduced form, respectively, and the derived equation $v = V_1 \cdot V_2/(V_1 + V_2)$, where v is the rate of oxidation of substrate, was tested by Kröger and Klingenberg using antimycin to decrease the value of V_2 [11]. Van Ark and Berden have shown [19] that HQNO can similarly be used. Indeed, the latter equation can be tested with any inhibitor of the QH_2 oxidase activity under the condition that the inhibition of the QH_2 oxidase activity is proportional to the binding of the inhibitor. In this case,

$$v_i = \frac{V_1 \cdot V_2(1-\bar{Y})}{V_1 + V_2(1-\bar{Y})}$$

where $\bar{Y}$ represents the saturation of the binding site with inhibitor and v_i the rate of substrate oxidation in the presence of inhibitor. This equation can be rearranged to

$$\frac{1}{\text{inhibition}} = \frac{v_0}{v_0 - v_i} = \frac{1}{\bar{Y}}\left(\frac{V_2}{V_1} + 1\right) - \frac{V_2}{V_1}$$

where v_0 is the rate in the absence of inhibitor. In Fig. 1 the results are shown of an experiment in

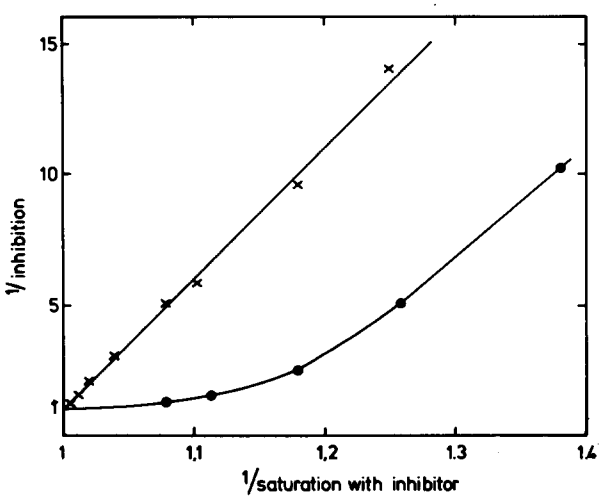

Fig. 1. Inhibition of the succinate oxidase activity of EDTA particles by HQNO and antimycin. The oxidation of 20 mM succinate by EDTA particles (0.5 mg/ml) was measured in a medium containing 0.25 M sucrose, 20 mM Tris-HCl buffer (pH 7.5), 0.2 mM EDTA, 4 μM cytochrome *c* and varying amounts of HQNO or antimycin. The oxygen uptake was measured with a Clark oxygen electrode. Temperature 30°C. The concentration of binding sites, determined fluorimetrically [13,19], was 0.35 nmol/mg and the K_D for HQNO 70 nM. For antimycin a K_D of $3.2 \cdot 10^{-11}$ M was assumed at high saturation [13]. The concentrations of the ethanolic solutions of the inhibitors were determined spectrophotometrically using for HQNO an absorbance coefficient of 10.45 $mM^{-1} \cdot cm^{-1}$ at 338 nm [19] and for antimycin a coefficient of 4.8 $mM^{-1} \cdot cm^{-1}$ at 320 nm [38]. The data are shown in a double-reciprocal plot. A hyperbolic inhibition results in a straight line with slope $V_2/V_1 + 1$ [19]. ×———× , HQNO; ●———●, antimycin.

which antimycin and HQNO were used as inhibitors of succinate oxidation. For HQNO the saturation is calculated from the independently determined K_D and the concentration of binding sites [19]. For antimycin the K_D is so small that only at high saturation was a correction for unbound antimycin made using a K_D of $3.2 \cdot 10^{-11}$ M [13]. It can be seen that the expected linear relationship between 1/inhibition and 1/saturation is obtained with HQNO, but not with antimycin. On the assumption that HQNO inhibits V_2 proportionally with the saturation of its binding site, the inhibition of V_2 by antimycin relative to the saturation with antimycin can be determined. This is shown in Fig. 2A. The clearly sigmoidal relationship between saturation and inhibition of the QH_2 oxidase activity can be analysed according to the model of Monod et al. [21], resulting in the data plotted in Fig. 2B. The values of α ([free antimycin]/K_R) were calculated from the K_D values at different levels of saturation as determined previously [13]. The value for *c* (the ratio of the K_D in the R (inactive) state and the K_D in the T (active) state) was also obtained from Ref. 13. The value obtained in this way for *n*, i.e., the number of interacting binding sites, is 8, very similar to the value previously reported. The value of *L*, i.e., the ratio of the concentration of T and R states present in the absence of inhibitor, is now lower than that previously reported, since in Ref. 13 no correction was made for the overcapacity of the QH_2 oxidase activity relative to the Q reductase activity (cf. Ref. 11).

The confirmation of the sigmoidal nature of the antimycin inhibition curve has some consequences: first, antimycin should not be used to test the pool function of ubiquinone, and secondly, antimycin bound to its binding site at low saturation (the T state according to the model used) does not inhibit electron transfer.

Comparison between the effects of HQNO and BAL + O_2

Evidence has recently been presented that the irreversible inactivation of the respiratory chain brought about by incubating particles with BAL + O_2 [22] is due to destruction of the Rieske Fe-S cluster [23], which is essential for electron-transfer activity [24]. The binding parameters for HQNO (and antimycin) are not affected by BAL treatment (see Fig. 3). Since the concentration of the Fe-S cluster can be measured by EPR spectrometry, the effect on the succinate oxidase activity of partial inactivation by BAL can be compared with that of partial occupation by HQNO of its binding site. In Fig. 4 it can be seen that saturation of the HQNO-binding site with HQNO has precisely the same effect as destruction of the Fe-S cluster. Thus both sites are part of a single asssembly of respiratory-chain carriers. The logical consequence is that no component with a pool function is involved between the two sites.

This is confirmed by the experiment shown in Fig. 5. After destruction by treatment with BAL of 81% of the Fe-S clusters as measured by EPR spectrometry, the residual activity was further in-

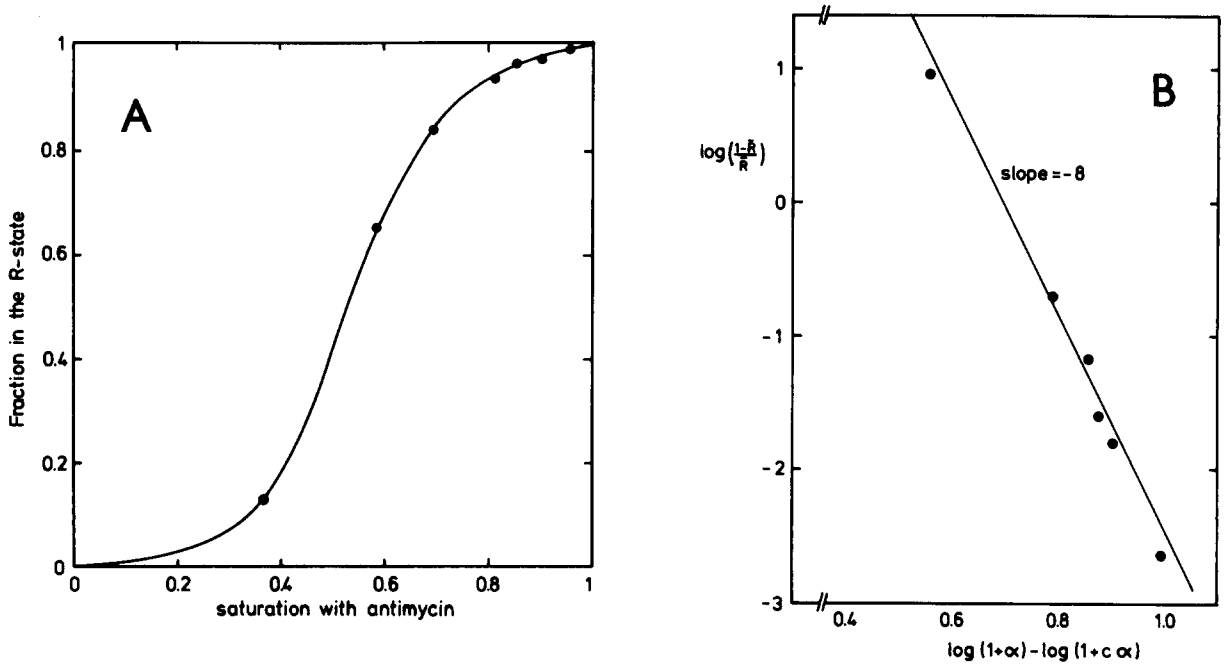

Fig. 2. Inhibition of the QH_2 oxidase activity of EDTA particles by antimycin. A. The percentage inhibition of the QH_2 oxidase activity at various levels of saturation with antimycin is calculated from the saturation with HQNO needed to obtain an equal inhibition of the succinate oxidase activity. The assumption is made that saturation with HQNO is proportional to inhibition of the QH_2 oxidase activity [19]. The inhibition state is called the R state. B. The parameters n and L of the model of Monod et al. [21] were determined using the equation $\log(1-\bar{R})/\bar{R}) = \log L - n[\log(1+\alpha) - \log(1+\alpha c)]$ (Ref. 13). The values for $\bar{R}$ are taken from A and the values for c and the dissociation constants at various levels of saturation (needed to calculate α) are taken from Ref. 13. Extrapolation to the ordinate gives $\log L = 5.6$; $n = 8$.

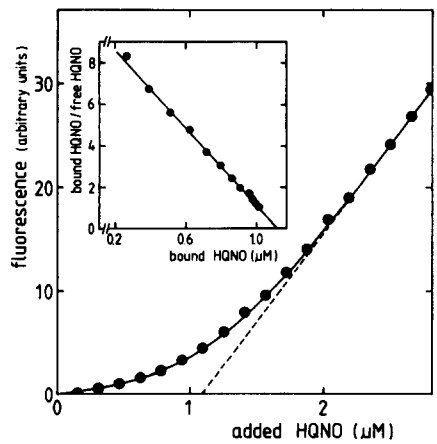

Fig. 3. Binding of HQNO to submitochondrial particles. EDTA particles were treated with BAL + O_2 so that all Rieske Fe-S cluster was destroyed, and then titrated with HQNO in a fluorimetric assay [19]. The concentration of particles was 3.67 mg/ml. The inset shows the Scatchard plot derived from the titration. The K_D equals 0.105 μM, the same as the K_D for nontreated particles at this protein concentration [19].

hibited by titration with HQNO. From the plot 1/inhibition versus 1/saturation, the V_2 and V_1 can be calculated. The ratio V_2/V_1 equals 3.4 in the absence of BAL treatment, and 0.72 after BAL treatment. Since V_1 is also slightly (15%) affected by the BAL treatment (cf. Ref. 22) we can calculate from these data that the V_2, as determined by HQNO, is inhibited by 82% ((V_2 after treatment)/(V_2 before treatment) $= 0.72 \cdot 0.85/3.4 = 0.18$) by the BAL treatment. This not only confirms Trumpower's finding [24] that the Fe-S protein is necessary for the oxidation of QH_2 but also shows that HQNO and BAL inhibit in the same linear segment of the QH_2 oxidase, although at different sites. Since in our present formulation of the respiratory chain [25] a semiquinone is an intermediate between the Fe-S cluster and the HQNO-binding site, it must be concluded that, if this model is correct, this semiquinone does not have a pool function, i.e., at the level of the

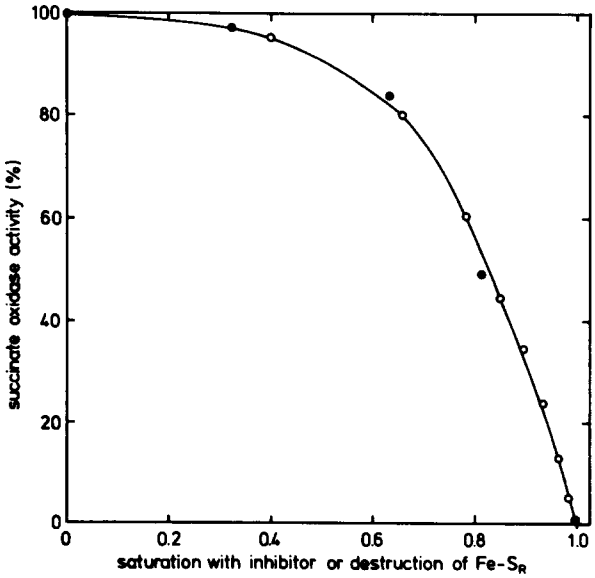

Fig. 4. Inhibition of the succinate oxidase activity of EDTA particles by HQNO and BAL ($+O_2$). The oxidase activity was measured as in Fig. 1 and the saturation with HQNO was calculated as described in the legend to Fig. 1. BAL treatment was carried out as described in the text and the destruction of the Rieske Fe-S cluster was measured by EPR spectroscopy [23]. The measured oxidase activity of BAL-treated preparations was corrected for the effect of the treatment on the succinate : Q reductase activity, measured as the 2,6-dichloroindophenol reductase activity. O——O, HQNO; ●——●, BAL.

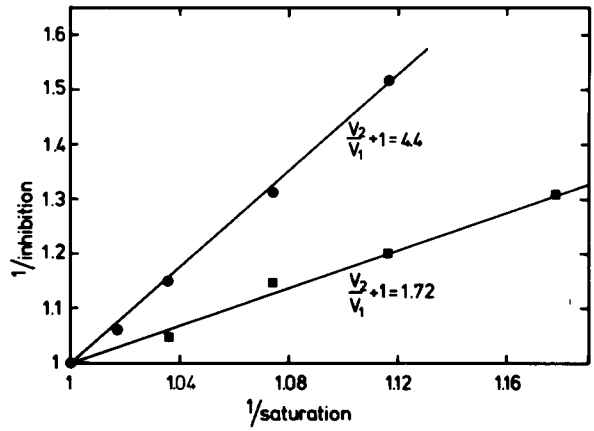

Fig. 5. Effect of BAL ($+O_2$) treatment of the HQNO-sensitive step in the succinate oxidase. The curve ●——● represents the inhibition by HQNO of the succinate oxidase activity of particles not treated with BAL; $V_2/V_1=3.4$. The curve ■——■ represents the inhibition by HQNO of the succinate oxidase activity of BAL-treated particles in which 82% of the Rieske Fe-S cluster is destroyed; $V_2/V_1=0.72$. Since the V_1 was inhibited 15% by the BAL treatment, it can be calculated that the HQNO-sensitive step (V_2) was inhibited 82% by the BAL treatment (see text):

semiquinone no rapid exchange between different respiratory-chain assemblies is possible.

Kinetics of reduction of cytochrome b in the presence of antimycin

Although BAL ($+O_2$) treatment of submitochondrial particles does not affect the reduction of cytochrome *b* by substrate [22], it causes inhibition of reduction of cytochrome *b* in the presence of antimycin [26] or HQNO. Extraction of the Fe-S protein from particles has the same effect [24,27]. In the presence of antimycin the reduction of cytochrome *b* in particles not treated with BAL is largely monophasic, unless cytochrome $c+c_1$ is partly reduced [28]. In particles in which the Fe-S cluster was destroyed to varying degrees, by treatment with different concentrations of BAL, the reduction of cytochrome *b* with succinate in the presence of a saturating amount of antimycin is biphasic, part of the cytochrome *b* becoming more slowly reducible (Fig. 6). Table I shows that the percentage of the cytochrome *b* that is still rapidly reduced runs parallel with the percentage of the Fe-S cluster that has survived BAL treatment. These data show that in the presence of antimycin an intact Fe-S cluster belonging to the same respiratory-chain assembly as cytochrome *b* is needed for the rapid reduction of the cytochrome *b*. The slow reduction of cytochrome *b* in respiratory-chain assemblies in which the Fe-S cluster is destroyed confirms the conclusion drawn above that the recently demonstrated semiquinone produced by the reduction of the Fe-S cluster by ubiquinol, and therefore sensitive to BAL but not to antimycin [29], does not function as a kinetically competent pool.

Partial pool function of free ubiquinone

All titrations of the oxidation of substrate with HQNO (or by BAL ($+O_2$) treatment) can be described by the equation $v=V_1\cdot V_2/(V_1+V_2)$. However, in some preparations, and especially with submitochondrial particles that had been frozen

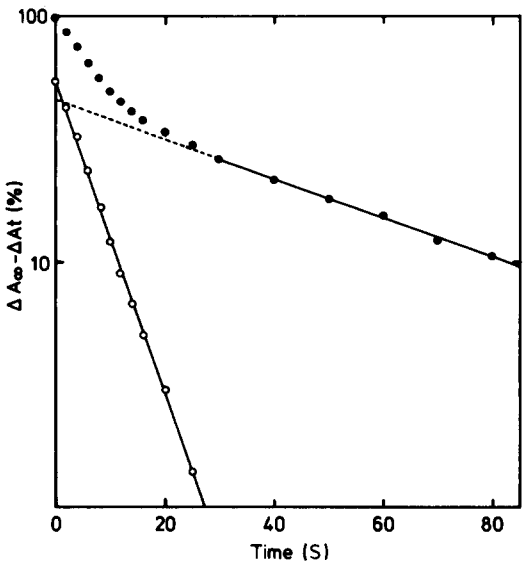

Fig. 6. Reduction of cytochrome *b* by succinate in the presence of antimycin after BAL ($+O_2$) treatment. To BAL-treated EDTA particles (2 mg/ml), suspended in the medium described in Fig. 1 but supplemented with 4 μM antimycin, 20 mM succinate was added and the absorbance change at 563 minus 577 nm followed. The temperature was 2°C. 48% of the Rieske Fe-S cluster in the particles was destroyed by treatment with BAL. The absorbance change is plotted semilogarithmically (●———●). The curve ○———○ is obtained by correcting the total absorbance change for the contribution of the slow phase. The rate constant for the rapid phase equals 0.14 s^{-1} and that for the slow phase 0.019 s^{-1}.

TABLE I

CORRELATION BETWEEN THE AMOUNT OF RESIDUAL RIESKE [2Fe-2S] CLUSTER, AFTER DESTRUCTION BY DIFFERENT CONCENTRATIONS OF BAL, WITH FRACTION OF CYTOCHROME *b* THAT IS RAPIDLY REDUCED BY SUCCINATE IN THE PRESENCE OF ANTIMYCIN

Submitochondrial particles were inactivated by shaking with different concentrations of BAL in the presence of oxygen, for 45 min at 25°C. Reduction of cytochrome *b* was measured as described in Fig. 6, the [2Fe-2S] cluster was measured by EPR spectrometry.

BAL (mM)	[2Fe-2S] (relative)	Fraction of *b* rapidly reduced (%)
0	100	89
1	51	54
2	20	20
4	0	0

and thawed a few times, the V_2 determined with HQNO varied with the substrate used, a lower value being found with NADH as substrate than with succinate. This is unexpected if electron transfer between NADH : Q oxidoreductase or succinate : Q oxidoreductase and the QH_2 : cytochrome *c* oxidoreductase is mediated solely by diffusible ubiquinone, since V_2 is defined as the rate of oxidation of QH_2 when all ubiquinone is present in the reduced form.

In order to investigate this discrepancy, dried submitochondrial particles were extracted with pentane a number of times and finally with pentane plus 10% acetone [30]. After the last extraction, no residual oxidation of succinate or NADH could be detected. The amount of Q extracted was determined and to construct Fig. 7 it was assumed that after the final extraction with pentane plus acetone all Q had been removed. Measurements on the rate of oxidation of duroquinol indicate that some Q, maximally 1% of the original amount, remained. With each of the preparations obtained, containing various amounts of ubiquinone, V_2 was determined by titration with HQNO, using both succinate and NADH as oxidizable substrate. From Fig. 8 it is clear that the value of V_2 is

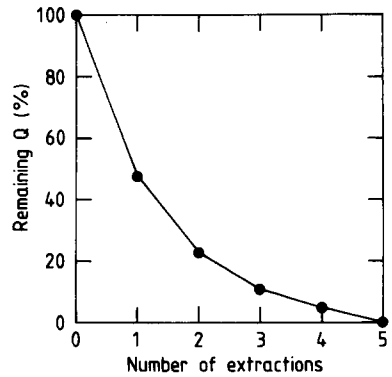

Fig. 7. Extraction of ubiquinone from ATP-Mg particles. ATP-Mg particles were washed twice with 150 mM KCl and lyophilised. The dry material was extracted with pentane and after each extraction the extracted ubiquinone was measured. For the last extraction pentane + 10% acetone was used. To calculate the ubiquinone content of the different particles obtained, it was assumed that after the last extraction (with pentane + acetone) all ubiquinone had been removed (however, see text). On this assumption the original content was 4.2 nmol Q/mg protein.

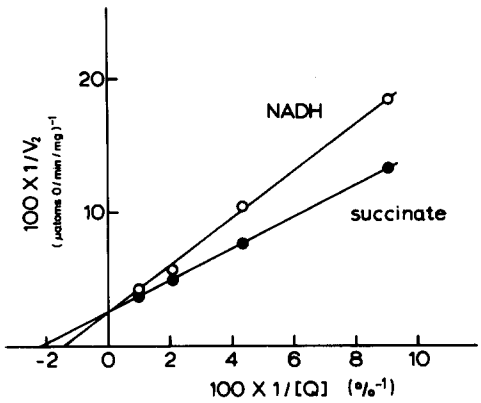

Fig. 8. Determination of V_2 for the oxidation of NADH and succinate. Using submitochondrial particles containing various amounts of ubiquinone (see Fig. 7) the V_2 and V_1 for the oxidation of succinate and NADH were determined using HQNO as inhibitor as described in Fig. 1. Only the dependence of V_2 on the ubiquinone content is shown.

different for the two substrates, this difference growing larger at lower Q content and decreasing to zero at infinite Q content. It seems, then, that the Q pool functions as the sole substrate for the Q-reducing and -oxidizing enzymes only at high Q concentrations. At lower levels of Q a direct interaction between the Q-reducing and Q-oxidizing enzymes must also be involved. The higher value for V_2 with succinate compared with NADH at finite Q concentrations may be due to the higher concentration of succinate:Q oxidoreductase, so that the collision rate of this dehydrogenase with the QH_2:cytochrome *c* oxidoreductase is greater than for the NADH:Q oxidoreductase. The fact that the K_m of ubiquinone for succinate dehydrogenase is lower than that for NADH dehydrogenase (unpublished data), so that more Q is sequestered by the succinate dehydrogenase, may also play a role.

Even if direct collision between Q-reducing and QH_2-oxidizing systems is possible in the absence of diffusible Q, bound Q is still required for electron flow from succinate to the QH_2:cytochrome *c* oxidoreductase. Successive extractions of ubiquinone by pentane resulted in a corresponding decrease in the rate of *b* reduction (measured at 2°C), not only in the absence of antimycin, but also in its presence, reaching zero after complete extraction. The apparent disagreement between these results and those of Ernster et al. [31], who found rapid reduction of cytochrome *b* in the presence of antimycin (but not in its absence) after extraction of the Q with pentane, is readily explained by the fact that the amount of Q remaining after pentane extraction [30] would be sufficient to catalyze the reduction of all the cytochrome *b* within the response time at the temperature used by these authors (24°C). Antimycin may also displace some Q from the antimycin-sensitive Q-binding site [3,4] and make it available to binding sites on succinate:Q oxidoreductase and the Fe-S protein in the QH_2:cytochrome *c* oxidoreductase.

Determination of the K_m of QH_2 for QH_2:cytochrome c oxidoreductase

Since it is clear from the data presented above that the K_m of QH_2 for the QH_2:cytochrome *c* oxidoreductase cannot be determined with succinate or NADH as substrate, we used duroquinol for this purpose.

When duroquinol is used as substrate, the rate of oxidation can be inhibited hyperbolically with HQNO (Fig. 9A and B), indicating pool-function kinetics. Although both V_1 and V_2 change with the concentration of duroquinol, the ratio V_1/V_2 is independent of this concentration and is a linear function of $1/[Q]$ (Fig. 10). The plot of $1/V_2$ against $1/[\text{duroquinol}]$ (Fig. 11) shows that the V_2 at infinite duroquinol concentration varies with the amount of residual ubiquinone, showing that Q is necessary for the oxidation of duroquinol. This requirement for ubiquinone cannot easily be detected when the overall rate of oxidation of duroquinol (v) is measured, since this is mainly determined by V_1 (independent of Q), V_2 becoming important only at low Q concentrations. This explains why Kröger and Klingenberg [32] and Boveris et al. [33] did not detect the requirement for Q and also why Weiss and Wingfield [34] did, since the preparation used by the latter was completely free of Q. The value of the K_m for Q calculated from this experiment is 14%, equal to 0.6 nmol Q/mg protein.

Ubiquinone is not necessary for the reduction of cytochrome *b* by duroquinol, although this reduction is sensitive to the combination of anti-

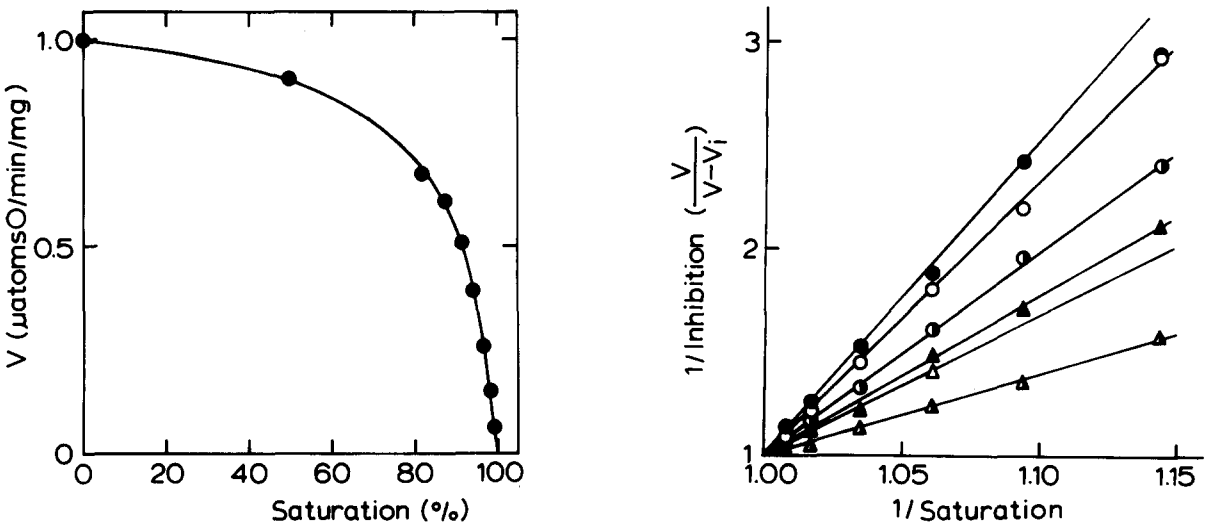

Fig. 9. A. Oxidation of duroquinol by ATP-Mg particles. The oxidation of 640 µM duroquinol by ATP-Mg particles (0.4 mg/ml) in 250 mM sucrose, 1 mM EDTA, 20 mM Tris-HCl buffer at pH 7.4 and 4 µM cytochrome *c* was measured in the presence of various amounts of HQNO. The HQNO-inhibition curve is hyperbolic. B. The plots 1/inhibition versus 1/saturation are given for particles containing different amounts of ubiquinone, the amount decreasing from top to bottom. For the nonextracted particles, the ratio V_2/V_1 equals 14 (top curve).

mycin and BAL treatment, like reduction by ubiquinol. Ubiquinone is also not necessary for the reduction of duroquinone by NADH, in the presence of antimycin. In our hands, antimycin only slightly (by 20%) affects the rate of reduction of duroquinone by NADH, but inhibits succinate oxidation by 50% (cf. Ref. 35). This difference could also be explained by a higher collision rate

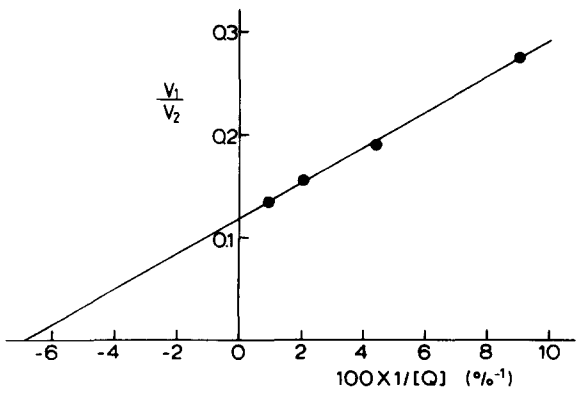

Fig. 10. Effect of ubiquinone content on the rate of oxidation of duroquinol. The experiments of Fig. 9 were repeated with various concentrations of duroquinol and with particles containing various amount of ubiquinone. The ratio V_1/V_2 is independent of the concentration of duroquinol. V_2 (but not V_1) changes with the ubiquinone content of the particles. From this plot the K_m of ubiquinone for the oxidase system measured by V_2 equals 14% of 4.2 nmol/mg, that is 0.6 nmol/mg.

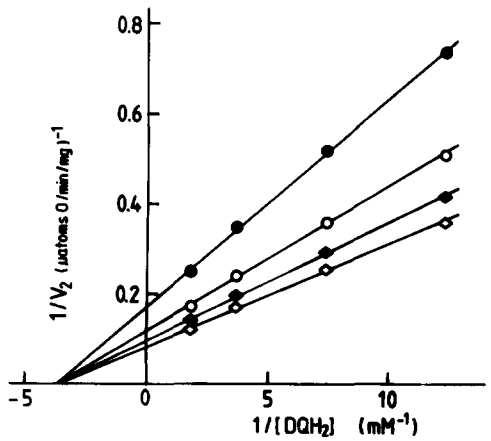

Fig. 11. Effect of duroquinol and ubiquinone concentrations on the rate of oxidation of duroquinol. The data for this figure are taken from the experiments described also in Fig. 10. The different lines represent particles with different amounts of ubiquinone. It is seen that the V_{max} for the oxidation of duroquinol depends on the ubiquinone content (cf. Fig. 10). The K_m of duroquinol for both the systems involved in the oxidation of duroquinol (V_1 and V_2) equals 280 µM.

of succinate dehydrogenase than of NADH dehydrogenase with QH_2: cytochrome *c* oxidoreductase (see above). With decreasing Q content the rate of reduction of duroquinone by NADH in the presence of antimycin decreases, but this effect disappears on extrapolation to infinite concentration of duroquinone. At the concentration of duroquinone (0.1 mM) used by Ruzicka and Crane [36], we also find a strong dependence of the rate of reduction of duroquinone on the residual ubiquinone. The reduction of duroquinone, both direct and that mediated by ubiquinone, is largely (95%) rotenone sensitive.

Discussion

It was shown earlier [13] that the shape of the antimycin-inhibition curve could not be fully explained by the fact that antimycin inhibits a site that is not rate-limiting [11], but that, in addition, antimycin has an allosteric effect. By using HQNO, we are now able to correct the antimycin-inhibition curve for the effect of the overcapacity of the QH_2 oxidase activity above that of the Q reductase activity. As a result of this correction, the value obtained for *L* is now smaller than previously, but the number of interacting subunits is not affected. A consequence of the special character of the antimycin effect (V_2 is inhibited sigmoidally by antimycin) is that, at low concentrations, antimycin can be bound to its site (in the T state according to the model of Monod et al. [21]) without inhibiting electron transfer. This is understandable if antimycin binds to a site other than that binding the semiquinone $Q_c^{\cdot -}$, as is suggested by the insensitivity to antimycin of binding a photoaffinity-labelled Q analogue to the enzyme [37]. It is more likely that antimycin binds close to the Q-binding site and induces a conformational change in the latter so that $Q_c^{\cdot -}$ is no longer bound. HQNO, which shows no allosteric effect and, just as antimycin, inhibits the formation of $Q_c^{\cdot -}$ (not shown) possibly binds to the $Q_c^{\cdot -}$ binding site itself. This would explain also the finding of Van Ark and Berden [19] that binding of antimycin excludes binding of HQNO.

According to the model favoured by us for electron transfer through QH_2: cytochrome *c* oxidoreductase (see Fig. 12), the site of interaction

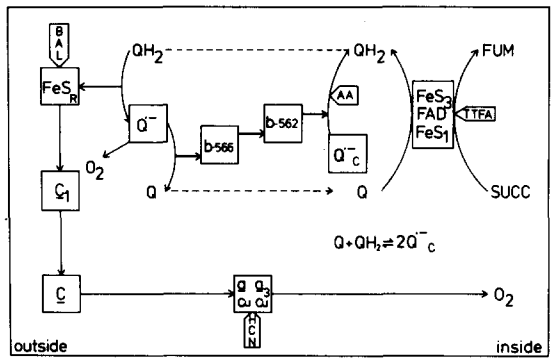

Fig. 12. Proposed scheme for the mechanism of oxidation of ubiquinol. In this scheme (cf. Ref. 25) the antimycin-sensitive $Q^{\cdot -}$ on the right-hand side is formed by reversal of the dismutation of ubisemiquinone. An alternative mechanism for this part of the scheme, involving a double Q cycle, is in preparation (De Vries, S., Albracht, S.P.J., Berden, J.A. and Slater, E.C., unpublished data).

of BAL is separated from the antimycin- and HQNO-binding site by a semiquinone formed by reducton of the Fe-S cluster.

This semiquinone, which has recently been detected by EPR spectroscopy [29], is the electron donor for cytochrome *b*. Since a semiquinone at neutral pH can only exist when it is stabilized by binding, a pool function for such a redox component is very unlikely. The experiments on the kinetics of the reduction of cytochrome *b* in the presence of antimycin confirm this idea: only those cytochrome *b* subunits that belong to a molecule of QH_2: ferricytochrome *c* oxidoreductase with an intact Fe-S cluster can be reduced rapidly. The slow reduction of cytochrome *b* in molecules in which the Fe-S clusters are destroyed may be due to direct reduction by QH_2.

The following evidence favours the views of Ragan and co-workers [14,15] that direct collision can take place between the Q-reducing and QH_2-oxidizing enzymes of the respiratory chain. (1) At low Q content, the values of V_2 are different for succinate and NADH oxidation. (2) Even with very low residual Q (less than 0.2 μmol/μmol cytochrome c_1), titration with HQNO indicates pool-function kinetics. (3) Nearly all the cytochrome *b* is reduced in a single fast phase in the presence of antimycin [25], although the redox level of Q changes markedly during the reduction

(Raap, Van Ark and Berden, unpublished results). If free Q were a necessary intermediate in the reduction of *b*, the kinetics of *b* reduction would be dependent on the redox state of Q.

It has been reported that duroquinol is a direct reductant of cytochrome *b* [35]. We have found, however, that this reaction is sensitive to the combination of BAL ($+O_2$) treatment and antimycin, which indicates that duroquinol reduces cytochrome *b* by the same pathways as ubiquinol. The fact that depletion of Q from the particles causes a lowering of the V_{max} of the oxidation of duroquinol indicates that the interaction of duroquinol with QH_2: ferricytochrome *c* oxidoreductase occurs via ubiquinone. It is, however, possible that there is also a direct interaction, but with a slow electron-transfer capacity. Since the V_{max} for reduction of duroquinone by NADH is independent of the presence of Q, direct interaction between duroquinone and NADH:Q oxidoreductase must take place. It is possible that duroquinone can bind to the same site as ubiquinone, although with lower affinity.

According to our present view, ubiquinone is involved in electron transfer between QH_2 and cytochrome *c* at two steps, the reduction of the Fe-S cluster and cytochrome *b*, and the oxidation of cytochrome *b*. It is possible that this second step, in which an electron is transferred from cytochrome *b* to a stable semiquinone in our scheme, cannot occur with duroquinone due to the absence of a stable semiquinone. The reverse of this step, the antimycin-sensitive reduction of cytochrome *b* by duroquinol, can occur in the absence of ubiquinone, as is evident from the fact that in BAL-treated Q-depleted particles cytochrome *b* can still be reduced by duroquinol, and this reduction is sensitive to antimycin. Since also the reduction of cytochrome *b* by duroquinol via the Fe-S cluster, in the presence of antimycin, is possible in the absence of ubiquinone, the need for ubiquinone for the overall electron transfer from duroquinol to oxygen can be most easily explained by the assumption that the ubiquinone is needed for the oxidation of cytochrome *b*, and this indicates that the step $DQH_2 + b^{3+} \rightarrow DQH^{\cdot} + b^{2+} + H^+$ is virtually irreversible, due to the lack of stabilization of the semiquinone form of duroquinone.

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