The pathway of electron flow through ubiquinol:cytochrome *c* oxidoreductase in the respiratory chain

Evidence from inhibition studies for a modified 'Q cycle'

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1. Cytochrome spectra of liver and heart mitochondria incubated under various conditions are presented to compare the effects of antimycin, colletotrichin and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) additions. 2. Under aerobic conditions, in State 4, in the presence of uncoupler or in the presence of cyanide, all three inhibitors caused oxidation of cytochromes *c* and *c*₁, but different changes in the spectra of the *b* cytochromes. Antimycin caused oxidation of a peak at 558 nm and reduction of peaks at 562 nm and 566 nm, whereas colletotrichin caused reduction of peaks at 558 nm and 566 nm and oxidation at 562 nm. HQNO had an effect on the spectra intermediate between those of the two other inhibitors. 3. Under aerobic conditions in the presence of 5 mM-succinate and 5 mM-fumarate, antimycin caused reduction of a peak at 566 nm and oxidation of a peak at 558 nm, whereas colletotrichin had the reverse effect and HQNO caused reduction of a peak at 562 nm. 4. Colletotrichin inhibition of the ADP-stimulated oxidation of glutamate + malate was enhanced by succinate addition and declined again with rotenone addition. Similar but smaller effects were seen with inhibition by antimycin and HQNO. 5. Cytochrome spectra are shown of the effects of ADP and uncoupler addition to stimulate respiration progressively. 6. The results are interpreted in terms of a modified 'Q cycle' [Mitchell (1976) *J. Theor. Biol.* **62**, 327–367] in which the three inhibitors are postulated to displace ubiquinone and ubisemiquinone specifically bound to cytochromes *b* on both sides of the membrane. 7. It is suggested that cytochromes *b*₅₅₈ and *b*₅₆₆ are the same *b* cytochrome located on the outer surface of the membrane, but binding ubisemiquinone or colletotrichin and ubiquinone or antimycin respectively. Cytochrome *b*₅₆₂ is postulated to be on the inner surface of the mitochondrial membrane and to bind either ubiquinone or ubisemiquinone. HQNO would bind to the reduced form of the cytochrome and colletotrichin to the oxidized form. 8. Sites for the locus of action of glucagon and the protonmotive force on electron flow are suggested.

The pathway of electron flow through Complex III of the respiratory chain (ubiquinol:cytochrome *c* oxidoreductase) has been the subject of considerable controversy. Three interrelated questions are at the centre of this debate. Firstly, how many species of *b* cytochrome are there and what is their function and mutual relationship (Wikström, 1973; Rieske, 1976; Wainio, 1977; Arutjunjan *et al.*, 1978)? Secondly, how is electron flow regulated by the protonmotive force (Erecinska *et al.*, 1973; Wikström, 1973; Papa, 1976; Papa *et al.*, 1981)? Thirdly, by what

Abbreviations used: HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; Complex III, ubiquinol:cytochrome *c* oxidoreductase.

mechanism(s) are protons ejected by the complex? Is it by a proton pump involving protein conformational changes (Papa, 1976; Papa *et al.*, 1981) or by a group-transfer reaction, as proposed by Mitchell (1976) in his 'Q cycle'? In addition to these three questions, with liver mitochondria an additional question arises with reference to the mechanism of stimulation of electron flow through Complex III by glucagon [see the preceding paper (Halestrap, 1982)].

The well-documented redox components of Complex III are two molecules of *b*-type cytochrome, one molecule of cytochrome *c*₁ and an iron-sulphur protein (Erecinska *et al.*, 1976; Rieske, 1976). The

two molecules of reduced cytochrome *b* exhibit three α -band absorption peaks at 562, 566 and 558 nm (Azzi & Santato, 1971; Lee & Slater, 1972; Wikström, 1971; Higuti *et al.*, 1975; Wainio, 1977). The peaks at 558 and 566 nm have been ascribed to the same *b* cytochrome, since b_{558} normally occurs as a shoulder on the b_{566} peak, both becoming more reduced under energized conditions or at elevated pH (Azzi & Santato, 1971; Wikström, 1971). Magnetic-circular-dichroism studies support this conclusion (Arutjunjan *et al.*, 1978). However, Higuti *et al.* (1975) have shown that cytochrome b_{558} can be reduced independently of b_{566} , supporting a separate identity of these components. Another cytochrome *b* component with a reduced absorption maximum at 560 nm has been reported in some mitochondria, but is not generally accepted as being important in the function of Complex III (Wikström, 1973; Wainio, 1977).

The sensitivity of the redox state of cytochrome b_{566} to the energy state of the mitochondria and the ambient pH have led to the proposition that it plays an important role in energy transduction and may be the site of coupling between the generation of the protonmotive force and respiratory-chain activity (see Chance *et al.*, 1970; Wikström, 1973; Papa, 1976). The mechanisms by which protons are pumped out of the mitochondria during respiration are not totally understood. For Complex III, two mechanisms have been proposed, the group-transfer reactions involving ubiquinone in a 'Q cycle' (Mitchell, 1976) or a 'vectorial Bohr shift' involving proton translocation resulting from protein-conformational changes (Chance, 1972; Papa, 1976; Von Jagow & Engel, 1980; Guerrieri *et al.*, 1981). The 'Q cycle' requires an involvement of ubisemiquinone in the electron flow within Complex III itself. Evidence is now accumulating that such radicals do exist and that ubiquinone is an essential part of functional Complex III (C. A. Yu *et al.*, 1977, 1978, 1980; L. Yu *et al.*, 1978; Yu & Yu, 1980, 1981). Furthermore, various inhibitors of electron flow in Complex III are ubiquinone analogues (Roberts *et al.*, 1978; Convent & Briquet, 1978; Trumpower & Haggerty, 1980) and may act at more than one site (Convent *et al.*, 1978; Izzo *et al.*, 1978).

In the present paper use is made of a novel inhibitor of the respiratory chain, collettotrichin (Foucher *et al.*, 1974; Suzuki *et al.*, 1976), in conjunction with other inhibitors of the respiratory chain, uncouplers and glucagon pretreatment of animals to provide further evidence for the validity of the 'Q cycle'. Furthermore, a scheme is proposed for electron flow through Complex III which accounts for both the effects of glucagon on the cytochrome spectra and the behaviour of the *b* cytochromes towards energization and a variety of respiratory-chain inhibitors.

Experimental

Materials

The sources of all chemicals and biochemicals are given in the preceding paper (Halestrap, 1982). Nigericin was obtained from Calbiochem (Behring Corp., Bishop's Stortford, Herts., U.K.) and was used as a 1 mg/ml stock solution in ethanol. Liver and heart mitochondria were prepared from female Wistar rats (250 g body weight) as described previously (Halestrap, 1975). Bovine serum albumin was present in the initial extraction medium at 10 mg/ml.

Methods

Measurement of cytochrome spectra at room temperature and -170°C were performed with a computerized split-beam spectrophotometer as described in the preceding paper (Halestrap, 1982). Exact conditions for each experiment are given in the legends to the individual Figures. Each set of spectra are representative of at least three similar experiments.

Rates of mitochondrial respiration were measured polarographically as described previously (Halestrap, 1978) and in the legend to Fig. 6 (below).

Results

Effects of antimycin, collettotrichin and 2-heptyl-4-hydroxyquinoline N-oxide on mitochondrial cytochrome spectra

In Fig. 1, data are shown of the effects of various respiratory-chain inhibitors on liver mitochondria oxidizing succinate in the absence of ADP or uncoupler (State 4). Antimycin, collettotrichin and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) all gave oxidation of cytochromes *c* and *c*₁ and reduction of *b* cytochromes [Fig. 1(a)(i–iii)]. However, careful inspection indicates subtle differences in the position of the cross-over. This is more clearly seen in the spectra performed at -170°C shown in Fig. 1(b), which show that antimycin (*b*, iii) caused an increase of a peak at 562.0 nm (-170°C) with a shoulder at 555.5 nm (-170°C). Collettotrichin (*b*, i) and HQNO (*b*, ii), however, gave peaks at both 562.0 nm and 555.5 nm. Comparison of the spectra at -170°C with those at room temperature suggests that these peaks largely represent cytochromes b_{566} and b_{558} . Subtraction of the antimycin spectra from these two spectra (iv, v) shows that in both cases a peak at 555.5 nm was more reduced by collettotrichin and HQNO than it was in the presence of antimycin, whereas a trough at 560.5 nm suggested that cytochrome b_{562} was more oxidized. The difference spectrum collettotrichin minus HQNO (*b*, iv) shows

Fig. 1. Effects of antimycin, collettotrichin and HQNO on the cytochrome spectra of liver mitochondria oxidizing succinate in State 4

Spectra were measured as described in the Experimental section. Liver mitochondria (approx. 10 mg of protein/ml) were incubated at 22°C in medium containing 125 mM-KCl, 2 mM-potassium phosphate, 2 mM-EGTA, 5 mM-succinate, 1 µg of rotenone/ml, 7 mM-Tris base and 10 mM-Mops (4-morpholine-propanesulphonic acid), pH 7.2. Oxygenation was achieved by addition of 5 mM-H₂O₂. For spectra at room temperature (a), samples were transferred to sample and reference cuvettes of the split-beam spectrophotometer and a baseline scan performed for correction of subsequent scans. Another scan was then performed after addition of (i) collettotrichin (20 µg/ml), (ii) HQNO (10 µM) or (iii) antimycin (1 µg/ml). The corresponding spectra measured at -170°C are shown in (b)(i)–(iii). The mitochondrial protein concentration in these experiments was 20 mg/ml (approx.). The spectra (iv)–(vi) represent difference spectra (iii–ii), (i–ii) and (i–iii) respectively in both (a) and (b).

that collettotrichin caused greater oxidation of this latter component than HQNO. Similar trends are seen in the spectra performed at room temperature, although in this case, in comparison with antimycin,

HQNO only caused reduction of b_{558} and not oxidation of b_{562} (a, iv) as was seen in the spectra performed at -170°C (b, iv). The data from Fig. 1 suggest that collettotrichin and HQNO inhibit at a site(s) distinct from antimycin and probably preceding the antimycin site such that an additional reduced b cytochrome with an absorption maximum at 558 nm appears.

Studies on uncoupled mitochondria support this conclusion and are shown in Fig. 2. Again all three inhibitors enhanced the reduction of the b cytochromes, but with very little oxidation of the c cytochromes, since these were already largely oxidized by the presence of uncoupler. Careful inspection of the spectra [Fig. 2(i)–(iii)] shows that both collettotrichin (iii) and to a lesser extent HQNO (ii) caused the appearance of a shoulder at 558 nm. This was confirmed by calculating the fourth-derivative spectra (see Halestrap, 1978), which bring out this shoulder as a peak (iv–vi). The fourth-derivative spectrum for collettotrichin inhibition (vi) indicates that b_{562} may be oxidized by collettotrichin, in agreement with the data obtained in State 4. Difference spectra are shown in Fig. 2(vii–ix), comparing the effects of the three inhibitors one with the other. These confirm that antimycin is the most effective at causing reduction of b_{566} and b_{562} (combined peak at 563.5 nm), whereas collettotrichin is the most effective at causing reduction of cytochrome b_{558} . However, detailed analysis of such difference spectra as shown in Fig. 1(b)(iv–vi) and Fig. 2(vii–ix) is not possible, since the changes seen will be highly dependent on the exact extent of inhibition of electron flow as well as on the locus of action of the inhibitor. Addition of HQNO to uncoupled mitochondria already inhibited with antimycin caused spectral changes consistent with reduction of c_1 (554), b_{558} and b_{562} and oxidation of b_{566} [Fig. 2(x)]. Addition of antimycin to uncoupled mitochondria inhibited with HQNO produced exactly opposite changes [Fig. 2(xii)]. However, addition of antimycin to mitochondria inhibited with collettotrichin caused spectral changes consistent with reduction of b_{562} , b_{566} and c_1 (554), but oxidation of b_{558} . Taken together, the spectra of Fig. 2 support distinct sites of action for all three inhibitors.

This conclusion is further strengthened and refined by the data of Fig. 3. In the spectra recorded in Fig. 3, mitochondria were incubated under aerobic conditions, but in the presence of 2 mM-cyanide, to inhibit respiration substantially (about 98%), but not totally, so that greatly diminished electron flow was occurring. Fumarate (5 mM) and rotenone were added to diminish greatly the reduction state of the cytochromes [see Fig. 3(iv) for the effects of rotenone]. Under these conditions antimycin induced a spectral peak at 564.5 nm (i), HQNO at 561.5 nm (ii) and collettotrichin at 559 nm.

Fig. 2. Effects on antimycin, collettotrichin and HQNO on the cytochrome spectra of liver mitochondria oxidizing succinate in the presence of uncoupler

Spectra were performed as described in the legend to Fig. 1, but with the addition of $5\mu\text{M}$ -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to uncouple respiration. The spectral changes induced by antimycin (i), HQNO (ii) and collettotrichin (iii) are shown, together with the corresponding fourth-derivative spectra (iv–vi). The spectra (vii)–(ix) represent the difference spectra (i–ii), (i–iii) and (ii–iii) respectively. Addition of HQNO to mitochondria already inhibited with antimycin produced the spectral changes shown in (x), whereas antimycin addition to mitochondria inhibited with collettotrichin or HQNO produced the changes shown in (xi) and (xii) respectively.

Addition of succinate to these inhibited mitochondria induced reduction of b_{566} in all cases (v–viii), although in the case of HQNO the effect was smaller. Cytochrome b_{558} was also reduced by succinate in the case of collettotrichin-inhibited mitochondria (viii) and to a lesser extent HQNO-inhibited mitochondria (vii). Only a very small effect was seen on antimycin-inhibited mitochondria (vi). Further addition of antimycin to mitochondria with succinate and collettotrichin resulted in oxidation of c_1 (554) and b_{558} and reduction of b_{566} (ix). A smaller

Fig. 3. Spectra of the effects of respiratory-chain inhibitors on aerobic mitochondria exposed to cyanide and fumarate

Mitochondria were incubated as described in the legend to Fig. 1, but in the absence of succinate and in the presence of 5 mM-fumarate and 2 mM-KCN. Spectra were performed after addition of antimycin (i), collettotrichin (ii) or HQNO (iii) to the sample cuvette. In (iv) the effect of rotenone ($1\mu\text{g}/\text{ml}$) on mitochondria exposed to fumarate is shown; all other conditions included rotenone. In spectra (v)–(viii) the effects of 5 mM-succinate addition to mitochondria exposed to fumarate and rotenone are shown in the absence (v) and presence of antimycin (vi), collettotrichin (vii) or HQNO (viii). In spectra (ix)–(xi) the additional spectral changes induced by antimycin addition to collettotrichin (ix)- and HQNO (x)-inhibited mitochondria or HQNO addition to antimycin-inhibited mitochondria (xi) are shown. Spectra (xii)–(xiv) are difference spectra performed in the presence of succinate and fumarate of antimycin – collettotrichin- (xii), antimycin – HQNO- (xiii)- and HQNO – collettotrichin (xiv)-inhibited mitochondria.

but similar effect was seen on addition of antimycin to HQNO-inhibited mitochondria (x). However, when HQNO was added to antimycin-inhibited mitochondria, only cytochrome b_{562} became more reduced. Comparison between the effects of the three inhibitors on the cytochrome spectra in the presence of cyanide, rotenone, succinate, and fumarate are shown in the difference spectra (xii–xiv). Antimycin caused oxidation of b_{558} and reduction of b_{566} relative to both collettotrichin (xii) and HQNO (xiii), whereas HQNO caused reduction of b_{562} and b_{566} (mean peak 563.5 nm) relative to collettotrichin (xiii).

These effects are more clearly seen in Fig. 4, where spectra of mitochondria incubated in the presence of succinate (5 mM), cyanide (2 mM) and

Fig. 4. Low-temperature spectra of the effects of antimycin, collettotrichin and HQNO on aerobic cyanide-inhibited mitochondria

Liver mitochondria were incubated with 5 mM-succinate, 5 mM-fumarate and 2 mM-KCN at 20 mg of protein/ml (approx.). Spectra were performed at -170°C as described in the Experimental section, after addition of $0.25\text{ }\mu\text{g}$ of antimycin/ml (i), $2\text{ }\mu\text{M}$ -HQNO (ii) or $25\text{ }\mu\text{g}$ of collettotrichin/ml (iii) to the sample cuvette. The fourth-derivative spectrum of (i) is given in (iv). The spectra (v)–(vii) represent difference spectra (ii–iii), (i–ii) and (i–iii) respectively.

rotenone ($1\text{ }\mu\text{g}/\text{mg}$ of mitochondrial protein) were performed at -170°C . Preliminary spectra at room temperature were used to find concentrations of the inhibitors which inhibited sufficiently to cause large changes in the spectra of the b cytochromes with little change in the state of cytochrome c reduction. If the added inhibitor was more potent than the cyanide at inhibiting electron flow, massive oxidation of cytochrome c occurred which masked the effects of the inhibitor on the b cytochromes. With these precautions taken, antimycin (i) was clearly seen to cause reduction of b_{566} (563.5 nm at -170°C) and b_{562} (559.5 nm at -170°C), the latter occurring as a shoulder which skews the peak of b_{566} and which is seen more clearly in the fourth-derivative spectra (iv). HQNO (ii) also caused reduction of these peaks, but in this case b_{562} was the more dominant peak. Collettotrichin (iii) caused reduction of b_{566} , oxidation of b_{562} and reduction of b_{558} (555.5 nm at -170°C). With antimycin (i) b_{558} appeared to become oxidized, whereas with HQNO, little effect on this cytochrome was apparent. All inhibitors caused some oxidation of cytochrome c (546.5 nm at -170°C) and c_1 (550.5 nm at -170°C), with collettotrichin causing the greatest oxidation of cytochrome c_1 . The difference spectra shown in (v)–(vii) confirm these differences between the inhibitors. HQNO was seen to maintain b_{562} and c_1 more reduced than with collettotrichin and b_{558} and b_{566} more oxidized (v). Antimycin maintained b_{562} , b_{566} and c_1 more reduced and b_{558} more oxidized than did collettotrichin, while causing b_{566} to be more reduced and b_{558} more oxidized than did HQNO (vii). Once again the data of Figs. 3 and 4 indicate different sites of action of the three inhibitors.

In Fig. 5 another set of conditions was used to help elucidate the sites of action of the three inhibitors. Heart mitochondria were allowed to become anaerobic in the presence of 5 mM-succinate, 5 mM-fumarate, rotenone ($2\text{ }\mu\text{g}/\text{ml}$) 1.5 mM-ascorbate and $50\text{ }\mu\text{M}$ -NNN'-N'-tetramethyl-*p*-phenylenediamine. Addition of antimycin at this point caused a small oxidation of cytochrome b_{558} and a substantial reduction of cytochrome b_{566} (i). However, addition of collettotrichin had exactly the opposite effect (ii) and caused substantial reduction of cytochrome b_{558} and very slight oxidation of cytochrome b_{566} . Additions of HQNO caused no detectable changes in the redox state of cytochromes b_{558} or b_{566} , but caused some reduction of b_{562} to occur (iii). In the presence of collettotrichin or HQNO, antimycin again caused oxidation of cytochrome b_{558} and reduction of cytochrome b_{566} (iv, v).

Effects of succinate and rotenone on the inhibition of respiration by antimycin, HQNO and collettotrichin

Foucher *et al.* (1974) noticed that when oxidation

Fig. 5. Effects of antimycin, collettotrichin and HQNO on the cytochrome spectra of anaerobic heart mitochondria

Heart mitochondria (10 mg/ml approx.) were incubated in the presence of 5 mM-succinate, 5 mM-fumarate, 1.5 mM-ascorbate, 50 μ M-*NNN'*-tetramethyl-*p*-phenylenediamine and 1 μ g of rotenone/ml until they became anaerobic. Spectra were performed at 22°C after addition of 1 μ g of antimycin/ml (i), 50 μ g of collettotrichin/ml (ii) or 10 μ M-HQNO (iii) to the sample cuvette and the re-establishment of anaerobiosis. The spectra (iv) and (v) show the spectral changes induced by further addition of antimycin to mitochondria previously exposed to collettotrichin and HQNO respectively.

of glutamate + malate by mitochondria in State 3 (ADP present) was inhibited by acetyl-collettotrichin, addition of succinate enhanced this inhibition. This was accompanied by oxidation of cytochrome *c*. The process was reversed by addition of rotenone, which caused reduction of cytochrome *c* and stimulation of respiration. Data in Fig. 6 demonstrate that succinate also enhances inhibition of glutamate + malate oxidation by collettotrichin and, to a lesser degree, by antimycin or HQNO. Addition of rotenone stimulated respiration in each case. In Fig. 7, spectral data are shown for antimycin inhibition of glutamate + malate oxidation in State 4 (i). Addition of succinate caused oxidation of *b*₅₅₈ and reduction of *b*₅₆₆ (ii), whereas further addition of rotenone reversed these effects (iii). These data confirm that the effect of succinate was to enhance antimycin inhibition. Similar experiments with HQNO gave

Fig. 6. Effects of succinate and rotenone on the inhibition of mitochondrial glutamate + malate oxidation by antimycin, collettotrichin and HQNO

Oxygen uptake was studied polarographically as described in the Experimental section. Glutamate (5 mM) + malate (2 mM) were the initial substrates, succinate (5 mM) being added at S and rotenone (0.1 μ g/ml) at R. Other additions were 2 mM-ADP as indicated and 0.25 μ g of collettotrichin/ml at C (i), 0.02 μ g of antimycin/ml at A (ii) or 0.5 μ M-HQNO at H (iii). Liver mitochondria were present at approx. 1 mg of protein/ml. Each trace is representative of three experiments on different batches of mitochondria.

spectral changes too small to be interpreted with certainty.

Effects of ADP and uncoupler on the cytochrome spectra of mitochondria oxidizing succinate

To elucidate the pathway of electron flow within Complex III more fully and so gain insights into the locus of action of the various inhibitors, the effects of stimulating respiration with ADP or uncoupler were studied (Fig. 8). The data presented indicate that stimulating respiration by either means caused the same spectral changes. A small stimulation of respiration with MgATP (which slowly produces ADP by endogenous ATPase activity) or low uncoupler concentrations caused the emergence of a broad oxidized peak at about 562 nm (i, iv). Increasing the uncoupler concentration or adding hexokinase and glucose to increase ADP production led to increased oxidation of this cytochrome peak with a shift in its centre towards 564 nm, and also oxidation of cytochromes *c/c*₁ (ii, iii and v-vii). Larger concentrations of uncoupler produced considerable oxidation of cytochrome *c/c*₁ (viii). This probably reflects the uncoupling of cytochrome oxidase enhancing cytochrome *c* oxidation, whereas the rate

Fig. 7. Effect of succinate on the spectral changes induced by antimycin in the presence of glutamate + malate

Liver mitochondria were incubated in State 4 as described in the legend to Fig. 1, but succinate and rotenone were replaced by 5 mM-glutamate + 2 mM-malate. Spectrum (i) shows the effect of adding 0.15 μ g of antimycin/ml to the reference cuvette, (ii) shows the subsequent spectral change induced by addition of 5 mM-succinate, and (iii) the further change induced by addition of 1 μ g of rotenone/ml.

Fig. 8. Changes in cytochrome spectra induced by stimulating respiration with ADP or uncoupler

Liver mitochondria were incubated in State 4 in the presence of 5 mM-succinate, 1 μ g of rotenone/ml and 10 mM-D-glucose. Spectra were measured after addition to the sample cuvette of 5 mM-MgATP (i), then 0.1 unit of hexokinase/ml (ii) and finally 2 mM-ADP (iii). A second experiment was performed with sequential additions of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone at 0.1 μ M (iv), 0.2 μ M (v), 0.4 μ M (vi), 0.6 μ M (vii) and 1.0 μ M (viii). Given alongside each spectrum is the increase in oxygen uptake (in ng-atoms of O/min per mg of protein) from the State-4 rate (35.5) caused by the addition. This was determined in a parallel experiment at the same protein concentration.

of reduction of cytochrome *c* by Complex III is already fully stimulated. Although the data do not allow detailed analysis of the site of the control of electron flow, the shift in the oxidized *b* cytochrome peak from about 562 nm to 564 nm is of obvious importance in the formulation of a pathway of electron flow within the complex.

Discussion

The data presented in Figs. 1–5 clearly show distinct sites of action of antimycin, HQNO and collettotrichin on electron flow through Complex III. When electron flow through Complex III proceeded at about 2% of maximal rate (aerobic cyanide inhibited, Figs. 3 and 4), 25% of maximal rate (State 4, Fig. 1) or at maximal rate (uncoupled, Fig. 3), there were differences in the nature of the changes in reduction state of the *b* and *c* cytochromes induced by the inhibitors. This is most clearly seen in Fig. 4, where spectra of aerobic cyanide-inhibited mitochondria were performed at -170°C .

All three inhibitors caused reduction of cytochrome b_{566} ; antimycin and HQNO also caused reduction of b_{562} , which collettotrichin appeared to oxidize, whereas HQNO and collettotrichin caused reduction of cytochrome b_{558} which antimycin

oxidized. The simplest rationalization of these results would be that collettotrichin and antimycin act at distinct sites, whereas HQNO acts on both sites. Two sites of action for HQNO have been suggested by others as a result of binding studies (Convent *et al.*, 1978) and rapid kinetic analysis of electron flow through the *b* cytochromes of HQNO-inhibited mitochondria (Izzo *et al.*, 1978).

However, consideration of the data of Fig. 3(i–iii) and Fig. 5 suggests that each inhibitor possesses a unique site of action. In Fig. 3(i–iii) mitochondria were exposed to cyanide, fumarate and rotenone, which might be expected to hold the cytochromes of the respiratory chain in a relatively oxidized form without any net electron flow. Under these conditions, antimycin caused the emergence of a peak at 564.5 nm, collettotrichin at 559 nm and HQNO at 561.5 nm, suggesting reduction of cytochromes b_{566} , b_{558} and b_{562} respectively. In Fig. 5, conditions were achieved for maintaining the cytochromes at a redox potential of about zero without electron flow [anaerobic conditions + succinate (5 mM) and fumarate (5 mM)]. Under these conditions, antimycin caused reduction of cytochrome b_{566} and slight

oxidation of b_{558} , whereas collettotrichin had the opposite effect. HQNO, however, caused reduction of b_{562} with no effect on b_{558} or b_{566} . The opposite effects of collettotrichin and antimycin on b_{558} and b_{566} suggest that b_{558} and b_{566} may be the same cytochrome which can be trapped in either form by the inhibitors. The reduced form, b_{558} , is stabilized by collettotrichin, whereas the reduced form, b_{566} , is trapped by antimycin. HQNO, on the other hand, appears to stabilize a separate entity, the reduced form of b_{562} . The shared identity of b_{558} and b_{566} is further supported by the effects of antimycin on collettotrichin-inhibited mitochondria under anaerobic conditions [Fig. 5(iv)] or aerobic conditions in the presence of cyanide [Fig. 3(i)]. The b_{558} peak induced by collettotrichin is reoxidized with the emergence of the b_{566} peak, as would be expected, since antimycin binds more tightly than collettotrichin (Foucher *et al.* 1974).

In order to account for the above findings, a scheme is proposed (Scheme 1) which utilizes the 'Q cycle' hypothesis of Mitchell (1976). The major features of this Scheme are that two distinct molecules of cytochrome *b* exist, one on each side of the membrane, and that both may bind ubiquinone, ubiquinol or ubisemiquinone. It is suggested that the free radical only exists bound to the *b* cytochromes, a view that is supported by three lines of evidence. Firstly, the free radical is extremely unstable and is likely to dismutate too rapidly to be a useful redox carrier molecule in the free form (see Rich, 1981). Secondly, there is evidence for a protein-bound free radical of ubiquinone from electron-spin-resonance studies (C. A. Yu *et al.*, 1977, 1978, 1980; L. Yu

et al., Wei *et al.*, 1981; Yu & Yu, 1981). Thirdly, ubiquinone analogues which may be covalently attached to proteins of Complex III by exposure to light specifically label the *b* cytochromes (Yu & Yu, 1980). The sites of action of antimycin, collettotrichin and HQNO are proposed to correspond to the various forms of ubiquinone bound to cytochrome *b*.

On the outer surface of the membrane it is proposed that the *b* cytochrome may bind ubiquinol and immediately be reduced with the production of a protein-bound semiquinone radical. This form of the *b* cytochrome has a reduced absorption peak at 558nm and is trapped in this form by collettotrichin acting as an analogue of the ubisemiquinone radical. The reduced cytochrome b_{558} -ubisemiquinone complex is thought to interact with the non-haem iron to which it loses an electron. The proximity of the non-haem iron to a ubiquinone radical is supported by the data of others (Trumpower, 1976; Bowyer & Trumpower, 1980; Bowyer *et al.*, 1980). Oxidation of cytochrome b_{558} would leave ubisemiquinone attached to the oxidized cytochrome, and subsequent oxidation of the radical and reduction of the *b* cytochrome would seem likely to occur. This would form a reduced cytochrome-ubiquinone complex which it is proposed is responsible for the absorption peak at 566nm. Antimycin is thought to compete with ubiquinone for this site, trapping the cytochrome in the reduced form. Support for this hypothesis comes from three observations: firstly, the spectral observations reported here; secondly, evidence from *Neurospora crassa* and yeast, where the antimycin titre is related to the mitochondrial

Scheme 1. Proposed pathway of electron flow through Complex III of the respiratory chain

Abbreviations used: Q, ubiquinone; QH•, ubisemiquinone; QH₂, ubiquinol; NHI, Rieske iron-sulphur protein; *b*, *c*, *c*₁, cytochromes *b*, *c*, *c*₁.

cytochrome b_{566} content (Von Jagow & Klingenberg, 1972; Roberts *et al.*, 1980); and thirdly from the observation that antimycin abolishes the electron-spin-resonance signal produced by the ubisemiquinone radical (Wei *et al.*, 1981). This latter observation would be expected if antimycin binds to all of the cytochrome b_{558}/b_{566} component, so displacing any bound ubisemiquinone.

The evidence for cytochromes b_{558} and b_{566} being different conformations of the same cytochrome is considerable. Firstly, both an increase in pH and addition of ATP to cyanide-inhibited mitochondria causes an increase in both absorption peaks (Azzi & Santato, 1971; Wikström, 1971). Secondly, only two molecules of cytochrome b are present in Complex III, whereas three absorption peaks are apparent (Erecinska *et al.*, 1976; Rieske, 1976). Thirdly, magnetic-circular-dichroism studies indicate that both 558 and 566 nm absorption peaks belong to the same species of cytochrome b (Arutjunjan *et al.*, 1978). Fourthly, the data provided here (Fig. 5) show that, in the reduced state with the redox potential held at about 0 mV, reduction of one of these two components by addition of collettotrichin or antimycin is always accompanied by apparent oxidation of the other component.

The oxidation of cytochrome b_{566} is proposed to occur by a transmembrane transfer of an electron to cytochrome b_{562} on the inner surface of the membrane. Ubiquinone could well be transferred in a concerted reaction and would then be reduced to ubisemiquinone by oxidation of cytochrome b_{562} . The oxidized cytochrome b_{562} -ubisemiquinone complex is proposed to be rapidly reduced by an electron from the respiratory substrate, either directly to the oxidized b_{562} -ubiquinol complex or via the reduced b_{562} -ubisemiquinone complex. HQNO is thought to inhibit at this point by acting as a ubisemiquinone analogue and trapping cytochrome b_{562} in the reduced state. This would account for all the observed spectral changes observed for this inhibitor. It is further proposed that collettotrichin may have a second site of action as a ubisemiquinone analogue, trapping cytochrome b_{562} in the oxidized form. Support for this comes from the spectra shown in Fig. 4, where, under cyanide-inhibited aerobic conditions, collettotrichin causes reduction of b_{558} and b_{566} , but oxidation of b_{562} . Furthermore, Foucher *et al.* (1974) report two phases in the inhibitory action of acetylcollettotrichin; a very rapid phase ($t_{\frac{1}{2}} < 1$ s), which is consistent with binding to cytochrome b_{558} on the outer surface of the membrane, and a slow phase ($t_{\frac{1}{2}} > 20$ s) consistent with binding to cytochrome b_{562} . The difference in $t_{\frac{1}{2}}$ could easily be attributed to the need for the inhibitor to cross the membrane to reach cytochrome b_{562} .

The location of cytochrome b_{566} on the outside of

the membrane and cytochrome b_{562} on the inside has been suggested by others (see Papa, 1976; Papa *et al.*, 1981). Such an arrangement suggests that the membrane potential should inhibit electron flow from cytochrome b_{566} to b_{562} and thus act as a control mechanism for electron flow at this point. Evidence from Papa's laboratory suggests that this is the case (Papa, 1976; Papa *et al.*, 1981). The role of the pH gradient in controlling the rate of electron flow is less clear. In Scheme 1 I have proposed the semiquinone to be bound as $QH^{\cdot}$ rather than $Q^{\cdot-}$, and thus protons will be released or taken up for electron transfers involving both ubiquinol oxidation and subsequent reduction. Whether this is the actual pathway taken or whether $Q^{\cdot-}$ is the semiquinone intermediate causing both protons to be taken up or released in the same redox reaction, depends on the relative pK values of the bound quinol and semiquinone. Whatever the exact pathway taken by the protons, they do provide a possible explanation for proton pumping in this region of the respiratory chain, as suggested by Mitchell (1976), and also could account in part for the pH-dependence of the redox mid-point potential of the b cytochromes (Urban & Klingenberg, 1969; Wilson *et al.*, 1972; Von Jagow *et al.*, 1978).

The effects of stimulating respiration with uncoupler or ADP (Fig. 8) are consistent with the proposed scheme. At low uncoupler concentrations, b_{562} oxidation is apparent and could be explained by an increased mitochondrial $[H^+]$ stimulating ubiquinol production from the ubisemiquinone-reduced cytochrome b_{562} complex. At higher concentrations, oxidation of cytochrome b_{566} becomes apparent and this is consistent with the decrease in membrane potential causing a stimulation of electron flow from cytochrome b_{566} to b_{562} . At very high uncoupler concentrations, enhanced oxidation of cytochrome c (caused by stimulation of cytochrome oxidase) masks any further effects on the b cytochromes.

There are four other observations which may also be explained by the Scheme presented. Firstly, there is the oxidant-induced reduction of cytochrome b in the presence or absence of antimycin (see Wikström, 1973), which any version of the 'Q cycle' can explain (Mitchell, 1976). Secondly, the Scheme allows for the observation that antimycin does not inhibit a single turnover of electrons through Complex III (Bowyer & Trumpower, 1981). Thirdly, the Scheme allows for the small but perceptible flow of electrons through the complex that is observed even in the presence of antimycin (Brown *et al.*, 1965). This would occur if a small proportion of the ubisemiquinone can migrate across the membrane from cytochrome b_{558} to b_{562} . Fourthly, the Scheme can explain why collettotrichin, and to a lesser extent antimycin and HQNO, are more effective inhibitors of respiration when succinate as well as glutamate +

malate are present (Fig. 6 and Foucher *et al.*, 1974). It is assumed that collettotrichin competes with ubiquinone for binding sites on the *b* cytochromes, whereas antimycin and HQNO compete for ubiquinol. Anything which increases the concentration of ubiquinol will decrease the availability of ubiquinone and ubiquinol for binding to the *b* cytochromes. Thus binding of antimycin, collettotrichin and HQNO will be subject to less competition by the natural substrate. It is known that succinate reduces the components of Complex III more effectively than glutamate + malate under uncoupled conditions (see, for example, Halestrap, 1982) and thus ubiquinol concentrations should also be elevated. An enhanced inhibition by collettotrichin, antimycin and HQNO would therefore be predicted by the proposed Scheme.

The final aspect of the model to be considered is the rate-limiting step in electron flow through the complex and thus the point stimulated in the respiratory chain of liver mitochondria after glucagon treatment of the rat. Elegant experiments have been performed recently with a hybrid electron-transport system comprising the photochemical reaction centre of *Rhodospseudomonas sphaeroides* and purified Complex III (Matsuura *et al.*, 1981). Single-flash kinetics indicate that the rate-limiting step in electron flow between cytochrome b_{562} and cytochrome c/c_1 involves a ubiquinone $\leftrightarrow$ ubiquinol interconversion. Rich (1981) has suggested that it is the formation of a ubiquinol-redox-protein complex that is likely to limit the rate of ubiquinol oxidation. In Scheme 1 it is suggested that this corresponds to the movement of ubiquinol from cytochrome b_{562} to b_{558} and its subsequent binding. Stimulation at this point would be expected to increase the reduction of cytochromes c , c_1 and b_{558} as is observed in liver mitochondria from glucagon-treated rats (Halestrap, 1982). Furthermore, the lipophilic nature of ubiquinone allows it to move within the membrane phospholipids, and any perturbation of these phospholipids might affect its mobility. Since previous data from this laboratory suggests that glucagon does exert an effect on the membrane phospholipids (see the preceding paper, Halestrap, 1982), such a hormone-induced change in ubiquinone motility seems feasible. In addition, the mitochondria from glucagon-treated animals showed enhanced inhibition by collettotrichin compared with control mitochondria (Halestrap, 1982). Since it is postulated that collettotrichin binds to cytochrome b_{558} , this lends credence to the view that a glucagon-induced change in phospholipid disposition also alters the binding of the natural substrate (ubiquinol) to this cytochrome. Indeed, perturbation of the phospholipids by ageing of mitochondria, addition of benzyl alcohol or treatment with phospholipase A_2 had the reverse effect to glucagon on collettotrichin

inhibition, spectral changes and respiratory-chain activity (A. E. Armston & A. P. Halestrap, unpublished work; Halestrap, 1982). In the preceding paper (Halestrap, 1982) it was pointed out that in some cases an additional site of glucagon action was suggested by an increased reduction of cytochrome b_{566} as well as b_{558} . This could merely reflect the extent of stimulation of ubiquinol transfer from cytochrome b_{562} to b_{558} , or otherwise be a result of stimulated electron flow from the ubiquinol pool on the substrate side of Complex III.

In conclusion, the present data support the modified version of the 'Q cycle' (Mitchell, 1976) shown in Scheme 1. This Scheme appears to fit all the available observations on the actions of inhibitors, changes in pH gradient and membrane potential and the effects of glucagon on the spectra of the *b/c* cytochromes. As such it provides a useful basis for future experiments.

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