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# INHIBITION OF ELECTRON TRANSFER FROM FERROCYTOCHROME *b* TO UBIQUINONE, CYTOCHROME $c_1$ AND DUROQUINONE BY ANTIMYCIN

G. VON JAGOW and C. BOHRER

*Institut für Physiologische Chemie und Physikalische Biochemie der Universität München, 8 München 2, Goethestr. 33 (G.F.R.)*

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SUMMARY

The effect of antimycin on (i) the respiratory activity of the KCN-insensitive pathway of mitochondria of *Neurospora* grown on chloramphenicol (chloramphenicol-grown) with durohydroquinone and succinate or NADH as substrate, (ii) the electron transfer from the *b*-type cytochromes to ubiquinone with durohydroquinone as electron donor as well as (iii) the electron transfer from the *b*-type cytochromes to duroquinone with succinate as electron donor in chloramphenicol-grown *Neurospora* and beef heart submitochondrial particles was studied. All experiments were performed in the uncoupled state.

1. The respiratory chain of chloramphenicol-grown *Neurospora* mitochondria branches at ubiquinone into two pathways. Besides the cytochrome oxidase-dependent pathway, a KCN-insensitive branch equiped with a salicylhydroxamate-sensitive oxidase exists. Durohydroquinone, succinate or NADH are oxidized via both pathways. The durohydroquinone oxidation via the KCN-insensitive pathway is inhibited by antimycin, whereas the succinate or NADH oxidation is not. The titer for full inhibition is one mol antimycin per mol cytochrome *b*-563 or cytochrome *b*-557.

2. The electron transfer from durohydroquinone to ubiquinone, which takes place in the KCN-inhibited state, does not occur in the antimycin-inhibited state.

3. The reduction of duroquinone by succinate in the presence of KCN is inhibited by antimycin. The titer for full inhibition is one mol antimycin per mol cytochrome *b*-566 or cytochrome *b*-562 for beef heart (or cytochrome *b*-563 or cytochrome *b*-557 for *Neurospora*).

4. When electron transfer from the *b*-type cytochromes to cytochrome $c_1$, ubiquinone and duroquinone is inhibited by antimycin, the hemes of cytochrome *b*-566 and cytochrome *b*-562 (or cytochrome *b*-563 and cytochrome *b*-557) are in the reduced state.

5. The experimental results suggest that the two *b*-type cytochromes form a binary complex the electron transferring activity of which is inhibited by antimycin, the titer for full inhibition being one mol of antimycin per mol of complex. The electron transfer from the *b*-type cytochromes to ubiquinone is inhibited in a non-linear fashion.

## INTRODUCTION

Antimycin is an inhibitor of respiration, its site of action is located between cytochromes *b* and $c_1$ [1]. It is known that, in the antimycin-inhibited state the *b*-type cytochromes are reduced, whereas the *c*-type cytochromes are in the oxidized state [2]. Furthermore, antimycin has an effect on the redox state of the *b*-type cytochromes. In the KCN-inhibited state with NADH or succinate as substrate only cytochrome *b*-562 is reduced, subsequent addition of antimycin causes the reduction of cytochrome *b*-566 [3, 4]. An effect of antimycin on cytochrome *b*-562 under this condition cannot be visualized, since it is already maximally reduced before antimycin addition. However, Wikström and Berden [5] have performed redox titrations of the *b*-type cytochromes with succinate in the antimycin-inhibited state which seem to indicate that besides a change of the redox state of cytochrome *b*-566, an apparent shift of the mid-potential of cytochrome *b*-562 from 40 mV to above 85 mV by antimycin occurs. Further support for an effect of antimycin on cytochrome *b*-562 is given by data from the red shift in the light-absorbance spectrum of reduced cytochrome *b*-562 [6–8] and from the shift of the EPR signal attributed to cytochrome *b*-562 [9].

The main peculiarity of the antimycin action is that the titer for full effect in every case: (i) inhibition of respiration [7, 8, 10, 11], (ii) reduction of cytochrome *b*-566 [12, 13] and (iii) inhibition of cleavage of Complex III [10, 14] is one mol of antimycin per mol of cytochrome *b*-566 or cytochrome *b*-562.

In this paper experiments were performed to see whether antimycin merely blocks the electron transfer from the *b*-type cytochromes to cytochrome $c_1$ or whether the electron transfer to ubiquinone or to an electron mediator directly interacting with the *b*-type cytochromes, as for instance duroquinone, is also inhibited. The various activities were tested and the antimycin titers for full inhibition were determined. Furthermore, the redox changes of the *b*-type cytochromes under these conditions were examined.

The experiments were performed with mitochondria and submitochondrial particles of *Neurospora* and beef heart. In *Neurospora*, by inhibition of the mitochondrial protein synthesis, it is possible to induce a second KCN-insensitive oxidase which leads to a branched respiratory chain [15]. As will be demonstrated in the first two sections of the results, the KCN-insensitive pathway of *Neurospora* can be used as a test for the existence or non-existence of electron flow from the *b*-type cytochromes to ubiquinone when durohydroquinone is used as electron donor. *Neurospora* is also useful for studying the redox changes of the *b*-type cytochromes, because in this species it is easier to differentiate between the *b*-type cytochromes than it is in beef heart [16, 17].

All experiments, except those involving the KCN-insensitive pathway, were performed in parallel with beef heart submitochondrial particles to ensure general validity of the results.

## METHODS

Hyphae of *Neurospora crassa* wild type (strain 74A), obtained from the Fungal Genetics Stock Centre (FGSC, Humboldt State College, Arcata, Calif.) were grown in suspension as described earlier [18].

Submitochondrial particles were prepared from mitochondria by ultrasonication (10 times at 5 s) in 0.1 M phosphate buffer, pH 7.0; after low-spin centrifugation the particles were sedimented by centrifugation at 100 000 $\times g$ for 1 h.

Ubiquinone extraction and reincorporation were performed with mitochondria according to the method of Ernster et al. [19] .

Oxygen consumption was measured amperometrically with a Clark-type electrode. The incubation medium generally used was 0.3 M sucrose, 10 mM Tris/acetate buffer, pH 7.2, 1 mM ethyleneglycol-bis(aminoethyl)-tetraacetic acid (EGTA), 1 mM phosphate, 0.5 mM $MgSO_4$ and 1 $\mu$M carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP).

The difference spectra of the cytochromes at room and low temperature were performed with a special split-beam spectrophotometer as designed by Klingenberg according to Chance [20].

The cytochrome contents were calculated with a correction method considering mutual interference of the cytochromes at both temperatures [17]. The extinction coefficients (reduced minus oxidized) used were: for cytochrome $aa_3$ $\Delta\varepsilon_{605-630\,nm}$ = 24.0 $mM^{-1} \cdot cm^{-1}$ [21], for cytochrome $b$ $\Delta_{560-575\,nm}$ = 23.4 $mM^{-1} \cdot cm^{-1}$ [22] and for cytochrome $c$ $\Delta\varepsilon_{550-542\,nm}$ = 18.7 $mM^{-1} \cdot cm^{-1}$ [23].

The content of the constituent ubiquinone was determined with an extraction method as described by Kröger and Klingenberg [24]. The content of the oxidized ubiquinone is determined after its extraction into organic solvent by reduction with

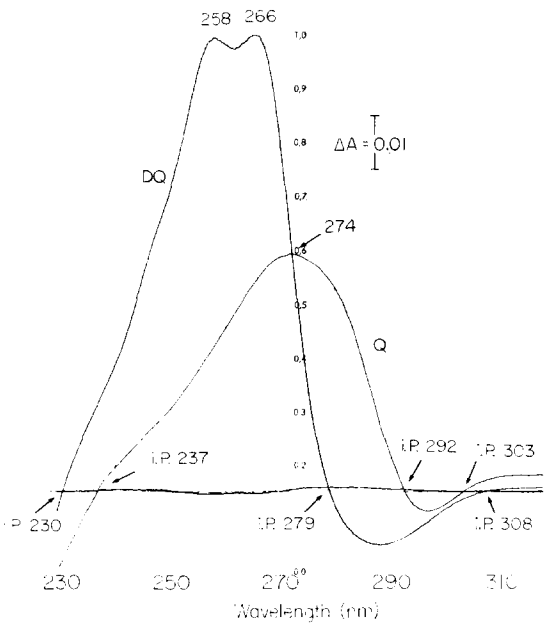


Fig. 1. The difference absorbance spectra of ubiquinone and duroquinone in organic solvent. Ubiquinone-9 (Q) and duroquinone (DQ) were dissolved in ethanol to a final concentration of 40 $\mu$M. The measurement was performed in a Cary spectrophotometer, model 118, with 1 cm light path. Reduction in the sample side was established by addition of $KBH_4$. The abbreviation used for isosbestic point is i.P.

$KBH_4$. Fig. 1 gives the difference spectra of duroquinone and ubiquinone in ethanol. As can be seen, redox changes of ubiquinone can be measured near its maximum at two isosbestic points of duroquinone in organic solution with the wavelength pair 279 and 308 nm. Control experiments showed that during the extraction procedure ubiquinone in oxidized or reduced form goes into the light-petroleum phase whereas durohydroquinone is quantitatively recovered in the methanol phase. Thus, a chemical reduction of ubiquinone by durohydroquinone in the light-petroleum phase cannot take place. Durohydroquinone in a molar excess of 20 : 1 does not interfere spectroscopically in the ubiquinone measurements performed at 279–308 nm. The measurements were performed with a Perkin-Elmer dual-wavelength photometer type 165. The extinction coefficient used for ubiquinone (oxidized minus reduced) was: $\Delta\varepsilon_{279-308\,nm} = 11.2\,mM^{-1} \cdot cm^{-1}$. Durohydroquinone was prepared from duroquinone in acetic solution by reduction with $SnCl_2$ and recrystallisation in ethanol [25]. The purity of the preparation was determined with the melting-point and spectrophotometric test. Protein was determined by the modified biuret method [26].

## RESULTS

### *The branching point of the respiratory chain of chloramphenicol-grown Neurospora mitochondria*

In a previous paper [15] it was reported that mitochondria of chloramphenicol-grown *Neurospora* possess a branched respiratory chain similar to that of the *mi*-1 mutant of *Neurospora*. From the antimycin titration studies it was assumed in analogy to the *mi*-1 mutant [17] that ubiquinone is the branching point in this chain.

A definite localization of the branching point was achieved by studying the dependence of the respiratory activities on the ubiquinone content of the mitochon-

TABLE I

INACTIVATION AND REACTIVATION OF RESPIRATORY ACTIVITIES BY MEANS OF UBIQUINONE EXTRACTION AND REINCORPORATION OF CHLORAMPHENICOL-GROWN *NEUROSPORA* MITOCHONDRIA

The extraction and reincorporation of ubiquinone was performed as described in Methods. Respiratory activity was measured with an oxygen electrode, using 1 mM NADH as substrate and 1 mM KCN, 2 mM salicylhydroxamate or both these inhibitors, as indicated. Chloramphenicol-grown WT is wild-type *Neurospora* grown in the presence of 4 g/l chloramphenicol.

| Type of mitochondria | Respiratory activity (μatoms 0/min per g protein) | | | |
|---|---|---|---|---|
| | – | +KCN | +Salicylhydroxamate | +Salicylhydroxamate +KCN |
| Chloramphenicol-grown WT | | | | |
| Fresh | 492 | 366 | 102 | 5 |
| Ubiquinone-depleted | 24 | 0 | 25 | 0 |
| Ubiquinone-incorporated | 227 | 147 | 80 | 20 |
| Wild type | | | | |
| Fresh | 525 | 0 | 520 | – |
| Ubiquinone-depleted | 33 | 0 | 30 | – |
| Ubiquinone-incorporated | 485 | 0 | 470 | – |

dria. The constituent ubiquinone was extracted and ubiquinone-9 was incorporated according to the method of Ernster et al. [19]. In the various preparations the respiratory activity of both branches and of the two pathways were measured separately, without addition of inhibitor, in the presence of KCN or in the presence of salicylhydroxamate, respectively.

The results obtained in a typical experiment are summarized in Table I. The respiratory activity was measured with NADH as substrate which is respired in *Neurospora* with a high rate by aid of an exogenous NADH-dehydrogenase [27]. The rate amounts to about 500 $\mu$atoms oxygen/min per g protein when both pathways are active. After ubiquinone depletion only about 5 % of the original activity is present. Ubiquinone incorporation leads to 46 % reactivation.

The respiratory activity of the second pathway, measured in the presence of KCN, is nearly completely inhibited by the ubiquinone depletion, and is reactivated to about 40 % after ubiquinone incorporation.

The respiratory activity of the normal pathway, measured in the presence of salicylhydroxamate, is decreased to 25 % after ubiquinone extraction and can be reactivated to 80 % by ubiquinone incorporation.

The control experiments with wild-type mitochondria give results similar to those obtained in mammalian mitochondria [28]. Ubiquinone depletion causes loss of 94 % of the original activity; by ubiquinone incorporation 92 % of the activity is restored. Addition of cytochrome *c* causes no increase in the respiratory activity (no shown). As expected, no activity can be measured in the presence of KCN. Salicyl-hydroxamate scarcely influences the respiratory activity of wild type mitochondria The experimental results show that the respiratory activities of the normal as well as of the KCN-insensitive pathway are ubiquinone dependent. The incomplete reactivation of the KCN-insensitive respiratory activity after ubiquinone incorporation may reflect partial denaturation of the KCN-insensitive oxidase by the extraction procedure. But the ubiquinone dependence of both respiratory activities shows that either ubiquinone itself or an unidentified component, located in the respiratory chain between ubiquinone and cytochrome *b*, perhaps an iron-sulfur center [29], represents the branching component. Fig. 2 summarizes the results in a scheme of the branched chain of chloramphenicol-grown *Neurospora*, not including the iron-sulfur proteins. It should serve for better understanding of the following experiments. The sequence of the two *b*-type cytochromes is not yet completely established, but kinetic experiments [30] favour the given arrangement. The inhibitor action marked by a double

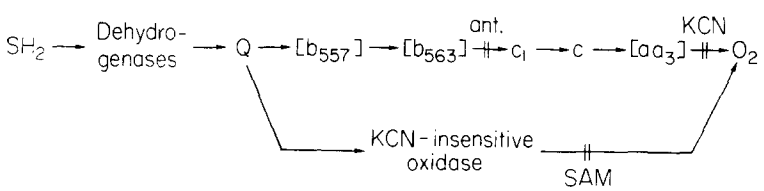


Fig. 2. Scheme of the respiratory chain of chloramphenicol-grown *Neurospora* mitochondria. Chloramphenicol-grown *Neurospora* mitochondria are prepared from hyphae which were grown in the presence of chloramphenicol. The content of components given in brackets is diminished as compared to wild type. The scheme does not include the iron-sulfur proteins. The abbreviations are: $SH_2$, substrate; Q, for ubiquinone; ant., antimycin; SAM, salicylhydroxamate.

line stands for the location of the block of electron transfer and does not indicate the inhibitor-binding site.

*Different effect of antimycin on durohydroquinone and NADH oxidation in the branched chain of chloramphenicol-grown Neurospora mitochondria*

Durohydroquinone is actively oxidized by beef heart mitochondria and the activity is sensitive to antimycin and KCN and independent of the constituent ubiquinone [31]. From studies with pigeon heart mitochondria it was suggested that durohydroquinone interacts directly with cytochrome *b* [32]. It seems reasonable to suppose that also in *Neurospora* mitochondria durohydroquinone reacts directly with cytochrome *b*.

As shown in Table II in *Neurospora* wild-type mitochondria durohydroquinone is as actively respired as NADH. As in mammalian mitochondria, the respiratory activity with both substrates is sensitive to antimycin or KCN and, as expected, insensitive to salicylhydroxamate.

In chloramphenicol-grown *Neurospora* mitochondria the durohydroquinone oxidation is 60 % as active as the NADH respiration. Both activities are only about 20 % inhibited by KCN and about 80 % inhibited by salicylhydroxamate. This shows that in the chloramphenicol-grown *Neurospora* mitochondria the main part of electron flow occurs via the KCN-insensitive pathway (cf. Fig. 2). The addition of antimycin reveals a striking difference between respiration with NADH or with durohydroquinone in the chloramphenicol-grown mitochondria. The oxidation of NADH is only 20 % inhibited by antimycin or KCN, whereas the activity with durohydroquinone is completely blocked by antimycin but not by KCN. This indicates that the reducing equivalents of durohydroquinone cannot reach the KCN-insensitive branch in the presence of antimycin, whereas those of NADH or succinate (not shown) can.

The titer for full inhibition of the durohydroquinone oxidation in the presence of KCN was determined by direct spectrophotometrical measurement of the durohydroquinone consumption. It was recorded by the increase in absorption at 270–284 nm. Full inhibition is reached at an antimycin concentration of about 0.14 $\mu$M

TABLE II

DIFFERENT EFFECT OF ANTIMYCIN ON DUROHYDROQUINONE AND NADH RESPIRATION OF CHLORAMPHENICOL-GROWN MITOCHONDRIA OF *NEUROSPORA*

Respiratory activity was measured with an oxygen electrode with 0.5 mM durohydroquinone or 1 mM NADH as substrate. Inhibitors added were: 2 nmol/mg protein antimycin, 1 mM KCN or 2 mM salicylhydroxamate as indicated. Chloramphenicol-grown WT is wild type grown in the presence of 3 g/l chloramphenicol. The experiment with wild type was performed as control.

| Type of mitochondria | Respiratory activity with ($\mu$atoms 0/min per g protein) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Durohydroquinone | | | | NADH | | | |
| | – | +Antimycin | +KCN | +Salicylhydroxamate | – | +Antimycin | +KCN | +Salicylhydroxamate |
| Chloramphenicol-grown WT | 329 | 0 | 266 | 65 | 579 | 455 | 455 | 104 |
| Wild type | 525 | 0 | 0 | 520 | 525 | 0 | 0 | 523 |

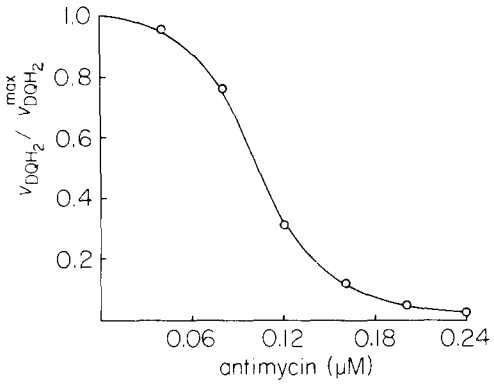


Fig. 3. Antimycin titration of the durohydroquinone oxidation of the KCN-insensitive pathway in chloramphenicol-grown *Neurospora* mitochondria. The rate of durohydroquinone ($DQH_2$) oxidation was measured with 0.1 mM $DQH_2$ as electron donor in the presence of 1 mM KCN at 25 °C with 0.75 mg protein/ml mitochondria of chloramphenicol-grown (1 g/l) *Neurospora* at 270–284 nm, light path 5 mm. Antimycin in ethanolic solution was added stepwise in 0.02 μM concentrations. The initial activity of the durohydroquinone oxidation ($v_{DQH_2}{}^{max}$) was 100 μmol/min per g protein.

which is equal to the actual concentration of cytochrome *b*-563 present in the incubation (cf. legend of Fig. 3). The shape of the titration curve, which is not linear, remains to be discussed.

The experimental results obtained by aid of the branched chain suggested that electrons can be transferred from durohydroquinone to ubiquinone via cytochrome *b* in the KCN-inhibited state but not in the antimycin-inhibited state. Therefore, in the following, the site of action of antimycin was studied in more detail.

*Inhibition of electron transfer from durohydroquinone to ubiquinone by antimycin*

The question whether the mitochondrial ubiquinone is reducible by durohydroquinone was studied by Boveris et al. [33] and by Kröger and Klingenberg [34, 35] and contradicting results were obtained. The direct optical methods applied by these authors for measuring the reduction of ubiquinone suffer from interferences by other components. Boveris' recording at 285–305 nm had an absorbance increase on every addition of durohydroquinone which had to be corrected and some interference by the δ-band of the *c*-type cytochromes. At 280–289 nm, the wavelengths used by Kröger, the oxidation of durohydroquinone interferes with the ubiquinone absorption, so that only small contents of durohydroquinone could be applied. To avoid such difficulties the redox state of ubiquinone is measured after extraction from the submitochondrial particles [19]. In the extracts thus obtained, the degree of reduction of ubiquinone can be determined without any interferences (see Methods).

Fig. 4 shows the dependence of ubiquinone reduction on increasing concentrations of durohydroquinone in the antimycin- and KCN-inhibited state. In the presence of KCN without durohydroquinone 10 % of ubiquinone is already reduced, probably by rests of endosubstrates. Maximum of ubiquinone reduction amounting to about 60 % is reached at 100 μM durohydroquinone. The relatively high concentration of durohydroquinone necessary for maximum reduction may be due to a small leak of electrons through traces of active KCN-insensitive oxidase, which in submito-

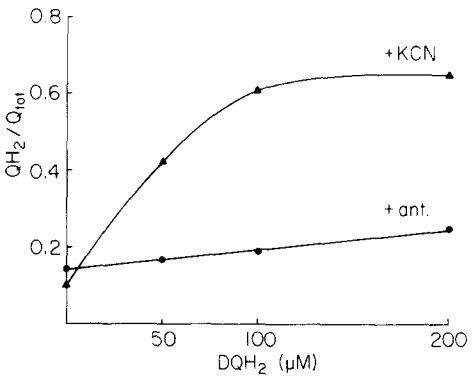


Fig. 4. The redox state of the constituent ubiquinone in dependence on the durohydroquinone concentration in antimycin- and KCN-inhibited state in chloramphenicol-grown submitochondrial particles of *Neurospora*. The experiment was performed with chloramphenicol-grown particles prepared from hyphae grown in the presence of 0.5 g/l chloramphenicol. The ubiquinone content was 6 μmol/g protein. The redox state of ubiquinone was determined by aid of the extraction method as described in Methods. The designations are: $QH_2$ for the content of reduced ubiquinone, $Q_{tot}$ for the total ubiquinone content. 2 mM KCN or 2 nmol/mg protein antimycin (ant.) were added as indicated. The incubation was performed at 25 °C for 1 min.

chondrial particles of chloramphenicol-grown *Neurospora* is almost fully inactivated.

In the antimycin-inhibited state about 15 % of the mitochondrial ubiquinone is reduced in the absence of durohydroquinone, and on its subsequent addition only about 5 % more is reduced. These data show that (i) the consituent ubiquinone can be reduced by durohydroquinone in the presence of KCN and (ii) that the ubiquinone reduction with durohydroquinone is blocked by antimycin.

In Fig. 5 the experiment of Fig. 4 is repeated with beef heart submitochondrial

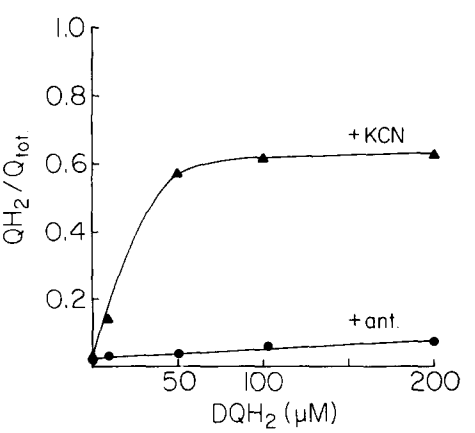


Fig. 5. The redox state of the constituent ubiquinone in dependence on the durohydroquinone concentration in antimycin- and KCN-inhibited state in beef heart submitochondrial particles. The ubiquinone content of the preparation was 10 μmol/g protein. The redox state of ubiquinone was determined by aid of the extraction method as described in Methods. The designations, additions and incubation conditions are as indicated in the legend of Fig. 4.

particles and similar results are obtained. In the presence of KCN durohydroquinone can reduce the constituent ubiquinone, whereas antimycin inhibits this reduction. In submitochondrial particles of beef heart there is nearly no reduction by endosubstrates and maximum reduction with KCN (60 %) is obtained at lower concentrations of durohydroquinone (about 50 $\mu$M). With antimycin the reduction increases only from 2 % to 8 %.

*The redox state of the b-type cytochromes in the KCN- and antimycin-inhibited state*

The influence of antimycin on the reduction of the *b*-type cytochromes in chloramphenicol-grown submitochondrial particles of *Neurospora* was studied by low-temperature absorbance spectroscopy. For control the redox state of ubiquinone

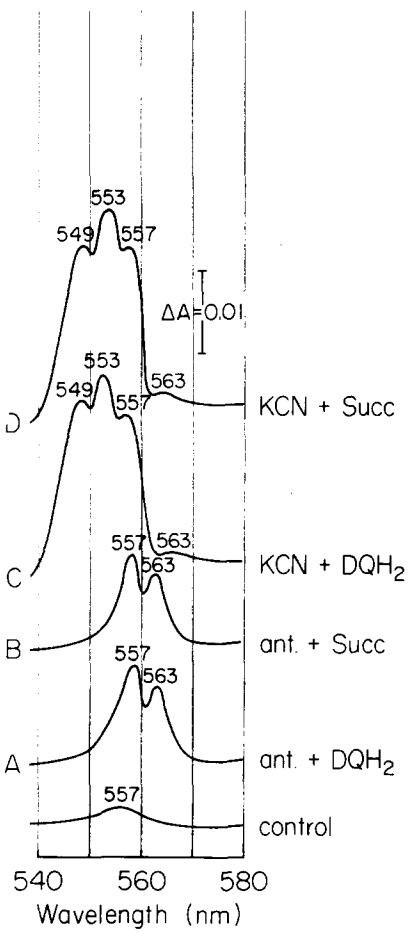


Fig. 6. Low-temperature difference absorbance spectra of the cytochromes of submitochondrial particles of chloramphenicol-grown *Neurospora* in the KCN- and antimycin-inhibited state. The experiments were performed with chloramphenicol-grown submitochondrial particles prepared from hyphae grown in the presence of 0.5 g/l chloramphenicol. The spectra were recorded in the states as indicated with oxidized state as reference. When indicated 5 mM succinate or 0.1 mM durohydroquinone $(DQH)_2$ and 1 mM KCN or 2 nmol/mg protein antimycin (ant.) were added.

was examined in parallel experiments by the extraction method. *Neurospora* particles have the advantage over beef heart particles that in the low-temperature spectra the *b*-type cytochromes can be better distinguished. The long-wavelength cytochrome *b* of *Neurospora*, formerly designated [17] cytochrome $b_T$, has its maximum at 563 nm at low temperature, the succinate-reducible cytochrome *b*, formerly cytochrome $b_K$, has its maximum at 557 nm.

In the antimycin-inhibited state with durohydroquinone as electron donor (cf. Fig. 6A) the two bands with maxima at 563 and 557 nm are visible. In the KCN-inhibited state with durohydroquinone as electron donor (cf. Fig. 6C) only cytochrome *b*-557 is mainly reduced, whereas the band of cytochrome *b*-563 can hardly be seen. The maximum at 553 nm and the shoulder at 549 nm are attributed to cytochrome $c_1$ [15].

With succinate as substrate similar spectra are obtained in the antimycin- (cf. Fig. 6B) or KCN-inhibited (cf. Fig. 6D) state. The control spectrum indicates that with KCN or antimycin (not shown) but without substrate addition only cytochrome *b*-557 is slightly reduced.

Table III gives the quantitative analysis of the cytochrome spectra and the data of the ubiquinone extracts. In the KCN-inhibited state both with durohydroquinone and succinate ubiquinone and cytochrome *b*-557 are mainly reduced, cytochrome *b*-563 remains chiefly in oxidized state. In the antimycin-inhibited state both *b*-type cytochromes are mainly reduced with durohydroquinone or succinate, but ubiquinone is in the oxidized state when durohydroquinone is used as electron donor, whereas with succinate it is in the reduced state.

Summarizing the data of the influence of antimycin or KCN on durohydroquinone and succinate oxidation two functional states can be differentiated: (i) in the KCN-inhibited state (cf. Fig. 7A) cytochrome *b*-557 is reduced by durohydroquinone to the same extent as with succinate. The electrons flow from the reduced cytochrome

TABLE III

THE REDOX STATES OF UBIQUINONE AND THE *b*-TYPE CYTOCHROMES OF CHLORAMPHENICOL-GROWN SUBMITOCHONDRIAL PARTICLES OF *NEUROSPORA* IN THE KCN- AND ANTIMYCIN-INHIBITED STATE

Addition of inhibitor and substrate as indicated in the legend of Fig. 4. The redox states of ubiquinone (Q) were determined by aid of the extraction method. The data of the redox states of the *b*-type cytochromes were calculated from the low-temperature spectra, performed in parallel, given in Fig. 4.

| State | Additions | | Redox level of (% reduction) | | |
|---|---|---|---|---|---|
| | Inhibitor | Substrate | Q | *b*-557 | *b*-563 |
| KCN-inhibited | KCN | Succinate | 75 | 80 | 7 |
| | KCN | Durohydroquinone | 65 | 70 | 3 |
| Antimycin--inhibited | Antimycin | Succinate | 75 | 70 | 80 |
| | Antimycin | Durohydroquinone | 10 | 65 | 78 |
| For control | KCN | None | 10 | 7 | 0 |

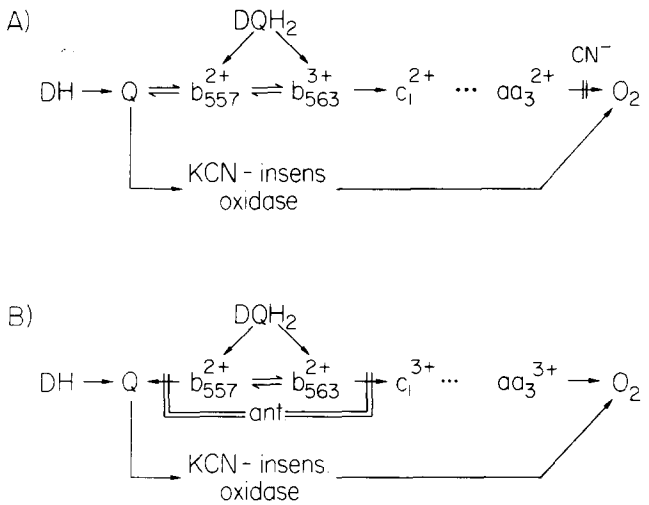


Fig. 7. Scheme of electron transfer in sumbitochondrial particles of chloramphenicol-grown *Neurospora* in KCN- and antimycin-inhibited state. (A) KCN-inhibited state. (B) Antimycin-inhibited state. The abbreviations are: DH, dehydrogenase; Q, ubiquinone; $DQH_2$, durohydroquinone; ant., antimycin.

*b* or succinate dehydrogenase via ubiquinone by aid of the KCN-insensitive pathway to oxygen; (ii) in the antimycin-inhibited state (cf. Fig. 7B) both *b*-type cytochromes are in the reduced state with durohydroquinone or succinate as electron donor. But the electrons of cytochrome *b* cannot be transferred to ubiquinone, therefore electrons do not flow from durohydroquinone via the KCN-insensitive branch to oxygen. Cytochrome $c_1$ is in the oxidized state, indicating that the electron flow from ferrocytochrome *b* to cytochrome $c_1$ is equally inhibited. With succinate, or with another substrate which is oxidized by a dehydrogenase as for instance NADH, the electrons flow via ubiquinone by aid of the KCN-insensitive branch to oxygen without any interference of the antimycin block.

As demonstrated in the scheme, a new site of inhibition of electron transfer by antimycin becomes apparent, that is the block between the ferrocytochromes *b* and ubiquinone.

*Inhibition of electron transfer from succinate to duroquinone by antimycin*

As shown by Ruzicka and Crane [36] duroquinone can serve as an electron acceptor for reducing equivalents supplied by NADH. They report that in electron-transport particles extraction of ubiquinone inhibits the duroquinone reduction with NADH and that reactivation can be obtained after the reincorporation of ubiquinone-10. Boveris et al. [33] have measured in pigeon heart submitochondrial particles the duroquinone reduction with succinate in the presence of KCN. They found an activity of 10–15 μmol/min per g protein which was sensitive to antimycin. This reaction was now reinvestigated in chloramphenicol-grown submitochondrial particles of *Neurospora* and beef heart.

The experiments were performed at 275–289 nm using the absorption band of duroquinone in aqueous solution. On addition of succinate in the presence of

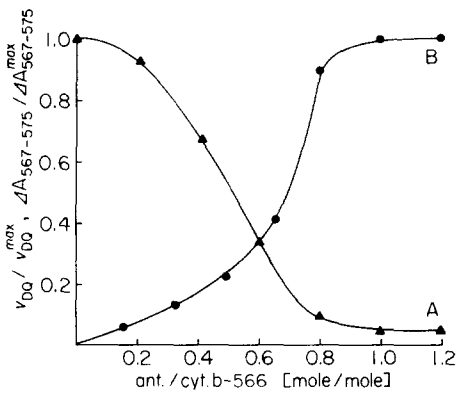


Fig. 8. Antimycin titration curves of the rate of duroquinone reduction and of the redox level of cytochrome *b*-566. Curve A, relative activity of the duroquinone reduction; Curve B, relative redox level of cytochrome *b*-566. The rate of duroquinone reduction was measured with 5 mM succinate and 0.1 mM duroquinone in the presence of 1 mM KCN at 25 °C with 0.3 mg protein/ml beef heart submitochondrial particles at 275–289 nm, light path 2 mm. Antimycin (ant.) in ethanolic solution was added stepwise in 0.04 $\mu$M concentrations. The initial activity of the duroquinone reduction ($V_{DQ}^{max}$) was 50 $\mu$mol/min per g protein. The increase in reduction of cytochrome *b*-566 was measured under the same conditions with 2.5 mg protein/ml beef heart submitochondrial particles at 567–575 nm, light path 5 mm. Antimycin in ethanolic solution was added stepwise in 0.2 $\mu$M concentrations.

KCN a linear decrease of the absorbance is recorded which corresponds to the reduction of duroquinone by succinate. The rates measured with chloramphenicol-grown submitochondrial particles of *Neurospora* and beef heart amount to 20–50 $\mu$mol/min per g protein. The activity can be titrated by antimycin as shown in Fig. 8, Curve A. This experiment was performed with beef heart particles. Nearly full inhibition of the activity is reached at an antimycin titer of about one mol antimycin per mol of cytochrome *b*-566 or *b*-562. The titration curve is not linear. The reduction of cytochrome *b*-566 during this titration can be followed in parallel experiments at 567–575 nm. In the KCN-inhibited state with succinate as electron donor cytochrome *b*-562 is in the reduced state whereas cytochrome *b*-566 is in the oxidized state. In the course of the antimycin titration cytochrome *b*-566 becomes more and more reduced (cf. Fig. 8, Curve B). When complete inhibition of the duroquinone reduction by antimycin is reached, it is maximally reduced. The leak of electrons through the cytochrome oxidase can be neglected. Cytochrome $c_1$ remains reduced to about 95 % during the whole titration (not shown). It was discussed earlier that the degree of reduction of cytochrome *b*-566 may reflect the amount of antimycin bound which is effective in the inhibition of respiratory activity [12, 37]. The non-linear courses of the antimycin titration curves of the rate of the duroquinone reduction and of the level of cytochrome *b*-566 reduction under this condition remain to be discussed.

## DISCUSSION

The experimental results show that: (i) when antimycin inhibits electron transfer from the *b*-type cytochromes to cytochrome $c_1$, the electron transfer to the constituent ubiquinone and to duroquinone is also inhibited; (ii) the titer for full inhibi-

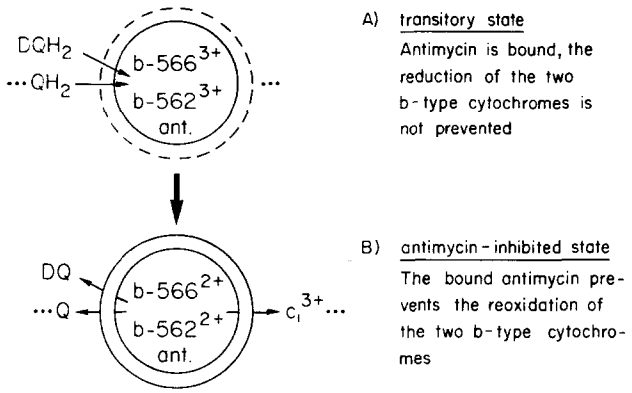


Fig. 9. A model for the mechanism of the antimycin action on cytochromes *b*. For detailed explanation see Discussion. Antimycin (ant.) in both cases is bound to the unknown binding site of a component which could be different from cytochrome *b*. The superpositioning of the two *b*-type cytochromes is not meant to give any indication of their arrangement in the respiratory chain.

tion in either direction is one molecule of antimycin for two molecules of cytochrome *b* (cytochrome *b*-562 and cytochrome *b*-566); (iii) the electron transfer from the *b*-type cytochromes to ubiquinone is inhibited in a non-linear fashion (whereas the electron transfer from the *b*-type cytochromes to cytochrome $c_1$ is inhibited in a linear fashion [35]).

For interpretation of the results a preliminary model for the mechanism of the antimycin action is given in Fig. 9. As known [38, 39], antimycin is bound to the *b*-type cytochromes both when they are in the oxidized (cf. Fig. 9A) or in the partially reduced state (cf. Fig. 7A). In Fig. 9A the "transitory state" represents the system in the absence of substrate. Antimycin is bound, yet the hemes of the *b*-type cytochromes prevail in the ferric form. On addition of an electron donor (as indicated by arrows in Fig. 9A) the *b*-type cytochromes are reduced instantaneously, antimycin becomes effective (cf. Fig. 9B). The reoxidation of the *b*-type cytochromes by cytochrome $c_1$, by ubiquinone or by duroquinone is prevented, as indicated by the broken arrows in Fig. 9B.

Since it is unlikely that one molecule of antimycin binds simultaneously to two different molecules of cytochrome *b*, it is assumed that the two *b*-type cytochromes form a binary complex of heme proteins, with an intimate interaction between the two molecules. This is marked in the model by a circle surrounding the two *b*-type cytochromes.

So far, no evidence exists that cytochrome *b*-562 and cytochrome *b*-566 form a binary complex of heme proteins. Some support for this hypothesis is given by the constant molar stoichiometry of the two *b*-type cytochromes which is held up in all species [41] as well as in the *mi*-1 mutant of *Neurospora* [42] and in chloramphenicol-grown *Neurospora* (v. Jagow, G., unpublished). Weiss and Ziganke [43] have isolated a cytochrome *b* preparation from *Neurospora* which is a dimeric protein, but further studies have to reveal whether it represents cytochrome *b*-563, cytochrome *b*-557 or both of them.

In a recent paper [44] a protein with an antimycin-binding site different from

cytochrome *b* was described. The actual binding site is, however, not yet known [39, 40]. The binding of antimycin to a component other than cytochrome *b* would demand that the complex consists of more than two molecules.

Whatever component carries the binding site, in our interpretation of the mechanism of inhibition the model shown in Fig. 9 requires that antimycin is bound in a linear fashion under all conditions. In contrast, Slater et al. [12, 38, 45] have published in various studies that the binding of antimycin can occur in either a cooperative or a non-cooperative manner. The data were derived from studies of the change of the redox state of the *b*-type cytochromes [12, 39] and from antimycin-binding studies [38]. The analysis of the latter is delicate since the dissociation constant of antimycin is very low. Slater admits [39]. that "the binding data are insufficiently accurate for a detailed analysis" so that, additionally, data of the antimycin inhibition of respiratory activities had to be taken into consideration [38]. But there is contradiction in the interpretation of the shape of the antimycin titration curves of those activities. Kröger and Klingenberg [35, 46, 47] describe these curves with an equation elaborated by aid of a kinetic model of the respiratory chain.

As will be discussed in the following, the non-linear inhibition of electron transfer between the *b*-type cytochromes and ubiquinone described in this paper cannot be explained by a kinetic model.

Three different classes of activities can be distinguished by the antimycin titration curves obtained so far: (i) respiratory rates which can give non-linear or linear titration curves depending on the kinetic conditions (respirations with a variety of substrates under different conditions); (ii) activities which give linear titration curves (the reoxidation of the constituent ubiquinone and the oxidation of durohydroquinone in the normal KCN-sensitive respiratory chain); and (iii) activities which give non-linear titration curves (the durohydroquinone oxidation via the KCN-insensitive pathway of chloramphenicol-grown *Neurospora* mitochondria (cf. Fig. 3) and the duroquinone reduction in all species as represented in Fig. 8A).

The activities classified under (i) and (ii) involve electron transfer from the *b*-type cytochromes to cytochrome $c_1$. The activities grouped under (iii) do not include this step. The increase of the degree of reduction of cytochrome *b*-566 which can be observed during antimycin titration in the presence of KCN (cf. Fig. 8B) satisfies the conditions of the (iii) activities.

The activities classified under (i) and (ii) are dealt with by Kröger et al. [35, 46, 47], those grouped under (iii) have not been analysed so far.

The durodydroquinone oxidation via the KCN-insensitive pathway should be kinetically comparable to the durohydroquinone oxidation via the cytochrome chains as described by Kröger. The rate-limiting step under our experimental conditions is the reduction of the *b*-type cytochromes by durohydroquinone. So, from kinetic aspects, a linear inhibition is expected, which is not observed (Fig. 3). In the case of the duroquinone reduction by succinate in the presence of KCN (cf. Fig. 8A) probably the rate-limiting step under our experimental conditions is the electron transfer from the *b*-type cytochromes to duroquinone, and precisely this step is inhibited by antimycin. Therefore, a linear titration curve is expected, in contrast to the experimental results.

The unexpectedly non-linear inhibition curves indicate that the electron transfer from the *b*-type cytochromes to ubiquinone and to duroquinone is inhibited in a

non-linear manner in contrast to the linear inhibition of electron transfer from the *b*-type cytochromes to cytochrome $c_1$ (in the case of the duroquinone reduction it should be considered that duroquinone might react with cytochrome *b* at the same site as ubiquinone).

The degree of reduction of cytochrome *b*-566 during antimycin titration in the presence of KCN may reflect the amount of antimycin which is effective in the inhibition of electron transfer from the *b*-type cytochromes to ubiquinone. In the course of the antimycin titration only part of the bound antimycin is effective in the inhibition of this step. Only when the binding site is saturated all the bound antimycin is fully effective, i.e. complete inhibition is reached.

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