Three functionally different cytochrome *b* redox centres in pigeon heart mitochondria

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1. Three functionally different cytochrome *b* redox centres, apparently of high metabolic activity, were detected in intact pigeon heart mitochondria: cytochrome *b(l)*, *b(m)* and *b(h)*, with maxima of absorption at 556.6 (State 5), 560.6, and 564.5 nm, respectively (α -bands, 77 K). 2. Cytochrome *b(l)* was reduced in the presence of either antimycin or HQNO (2-heptyl-4-hydroxyquinoline *N*-oxide). The absorption maximum was shifted by dithionite, cyanide, *NNN'*-tetramethyl-*p*-phenylenediamine + ascorbate, HQNO and antimycin. The spectra obtained on simultaneous or successive addition of HQNO and antimycin favoured the assumption of a common binding site for the two inhibitors. 3. Cytochrome *b(m)* was reduced in the presence of HQNO, but not in the presence of antimycin. No shifts of absorption maximum were observed. 4. Cytochrome *b(h)* was reduced in the presence of antimycin. HQNO was unable to cause reduction of this cytochrome by endogenous substrates. The absorption maximum was shifted to lower wavelength by organic solvents. It was inseparable from that of cytochrome *b(m)* in the presence of 0.4% ethanol. 5. The pattern of reduction in the presence of HQNO or antimycin demonstrates the functional difference of the three redox centres and appears incompatible with a linear respiratory chain.

The function of cytochrome *b* in the mitochondrial respiratory chain has been a subject of controversy for many years, because this function obviously is very complex and furthermore is highly dependent on the integrity of the preparations, as already realized in the 1950s [for a review, see Wainio *et al.* (1968)]. Different cytochrome *b* redox centres have been identified by means of spectrometry and redox titrations of preparations ranging from intact mitochondria to Complex III [for a review, see Wikström (1973)]. The question as to whether cytochrome *b* purified from Complex III is a single haemoprotein has not been completely resolved (Weiss, 1976; von Jagow *et al.*, 1978). It is, however, generally accepted that its properties are highly influenced by the environment in the membrane (e.g. Yu *et al.*, 1979). In the present paper the term 'redox centre' is used because the observed spectroscopic species of cytochrome *b* may owe their properties to different environments. Three absorption bands of cytochrome *b* in the region

556–566 nm at room temperature were first described by Chance (1958), but the low-wavelength band is most often considered a shoulder on the high-wavelength band rather than ascribed to a separate compound (Wikström, 1973). A number of mechanisms for the electron transfer in Complex III have been proposed (Baum *et al.*, 1967; Wikström & Berden, 1972; Mitchell, 1975; Rieske, 1976; Malviya *et al.*, 1980). None of these involve, however, three cytochrome *b* redox centres.

Antimycin affects the spectrum of reduced cytochrome *b* by causing a red shift (Pumphrey, 1962) and inhibits electron transfer from cytochrome *b* to *c*₁. Many mechanisms have been proposed for the action of antimycin [for reviews, see Slater (1973); Rieske (1976)]. The inhibitory action of HQNO resembles that of antimycin, and the experiments of van Ark & Berden (1977) and of Nijs (1967) suggest a common site of binding of the two inhibitors.

The present paper demonstrates that three functionally different cytochrome *b* redox centres exist in intact mitochondria. They appear to react in a non-sequential fashion. Spectrometric observations further support the assumption that HQNO and antimycin bind to the same site, probably located

Abbreviations used: HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

on or in the vicinity of the redox centre designated cytochrome *b(l)* in the present paper.

Experimental

Preparation of mitochondria

Pigeon heart mitochondria were prepared as described previously [Rasmussen (1969), cf. Tyler & Gonze (1967)]. The method involved very gentle homogenization, followed by incubation of the homogenate with bacterial proteinase for 10 min at 0°C (subtilisin A, obtained by courtesy of Novo, Copenhagen, Denmark). The amount of proteinase was 2 mg/g of tissue and the total volume for one heart was 80 ml. The preparation medium was 225 mM-mannitol / 75 mM-sucrose / 1 mM-Tris / 50 μ M-EDTA, pH 7.35. Young pigeons were the preferred starting material since their hearts required less vigorous homogenization.

The mitochondria were isolated and washed in four centrifugations lasting 7 min 40 s each (effective time). The rates were 8500, 1700, 8000 and 8000 rev./min respectively, and the tubes were filled maximally in all centrifugations ($r_{\min} = 5.6$ cm, $r_{\max} = 10.6$ cm). Centrifugal data are reported as recommended by Rasmussen (1973). The Sorvall RC-5 centrifuge, equipped with the SS-34 rotor (DuPont Company, Newton, CT, U.S.A.), was improved in temperature and time controls and the rate of rotation was measured with an accurate rate-meter. The temperature during centrifugation was 4°C. All loosely sedimenting material and floating lipids were carefully removed during the preparation. The yield of mitochondrial protein was consequently low (2–3 mg/g of tissue).

Electron micrographs showed a very homogeneous preparation devoid of particles other than mitochondria. The mitochondrial preparation was kept at 0°C at a concentration of 5–10 mg/ml for maximally 6 h. All water used was prepared from tap water which was filtered through activated carbon and distilled once in glass and twice in quartz equipment.

Protein was determined by a method based on staining with Amido Schwartz (Schaffner & Weissmann, 1973) with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as standard. This method gave analytical values close to 80% of the values obtained by a biuret method (Gornall *et al.*, 1949), corrected for turbidity and interference from medium components.

The instrument

Absorption spectra at 77 K were measured with a dual-wavelength spectrophotometer built in this laboratory and exhibiting some design features that

Fig. 1. Functional diagram of the dual-wavelength spectrophotometer

The character of the signals in the output section is indicated by A (analog voltage) or D (parallel digital signal).

will be briefly described (Fig. 1). More detailed descriptions of this type of instrumentation are given by, for instance, Chance (1957) and Garland *et al.* (1976).

The optical system was constructed around the two grating monochromators (type D330; Hilger and Watts Ltd., London N.W.1, U.K.). It included no moving parts, as the light was 'chopped' by rotating perforated discs placed at the entrance slits of the monochromators. Extremely low cross-talk between the channels and very high stability of the system was obtained with this design. A small dark period (about 1 ms) between the light periods (about 9 ms) apparently improved the stability of the photomultiplier's sensitivity. The two monochromatic beams were aligned to pass through the same part of the cuvette by a system of lenses and mirrors.

The wavelength drives of the monochromators were equipped with encoders and stepper motors. A digital electronic system effected readout and automatic adjustment of the wavelengths with a resolution of 0.1 nm.

The laminated construction of the cuvettes consisted of windows of polycarbonate sheet (2 mm thick) and a central part of black aluminium sheet (2 mm thick). A slot, milled in this central part, formed the cuvette cavity. During the measurements the cuvette was mounted in an unsilvered Dewar flask filled with liquid nitrogen to a level below the horizontal light path. The aluminium part of the cuvette immersed into the nitrogen, thus the cuvette content was kept at a temperature close to 77 K. A small heating element on the outside of the Dewar flask prevented frost formation.

The analog electronic system was rather conventional. It included a preamplifier, connected directly to the anode of the photomultiplier (type 9558B; EMI, Hayes, Middx., U.K.), two track and hold amplifiers, controlled by the light choppers and used for separation of the channels, a dynode feedback system for the reference channel, a logarithmic amplifier system for the scanning channel, and a difference amplifier, the function of which is described below. The logarithmic amplifier and the difference amplifier were equipped with calibrated gain and zero offset adjustments.

Scannings were performed as dual-wavelength scannings, i.e. the reference wavelength was fixed while the scanning wavelength was varied at a constant rate. The baseline, measured on a fully oxidized system, was stored in the digital baseline memory and, after conversion, subtracted from all later scannings by the difference amplifier. The output of the difference amplifier was connected to the x - y recorder and to the 10-bit converter of the computer. The baseline memory and the computer were addressed with the digital wavelength signal which, after conversion, was also registered by the x -channel of the recorder. The microcomputer (SPC 1; Dansk Data Elektronik, Herlev, Denmark) read the analog signal once per 0.1 nm. The resolution of this reading corresponded to at least 14-bit resolution of the signal from the preamplifier owing to the amplification and zero offsets in the analog system.

The time constant of the analog amplifier system was 0.1 s; the scanning rate was 1 nm/s. Model calculations showed that absorption maxima very reproducibly were delayed by one time constant. Thus all wavelength data have been corrected by 0.1 nm. The half-width of the monochromatic light was maximally 0.5 nm. A small mercury arc lamp (Ultra-violet Products Inc., San Gabriel, CA, U.S.A.) was used for calibration of the wavelength settings of the monochromators before and after each experimental series. During a 2 h warming-up period the monochromators exhibited a 0.2 nm drift in wavelength calibration, presumably because of heat radiation from the lamp.

Experiments

The reaction medium was 225 mM-mannitol/75 mM-sucrose/20 mM-Tris/0.5 mM-EDTA/10 mM-phosphate, pH 7.35. Inhibitors, substrates etc. were all present in the reaction medium before addition of mitochondria. Antimycin was added to the empty incubation tube as an ethanolic solution. The ethanol was evaporated under nitrogen and reaction medium added, followed by vigorous mixing. Incubations usually lasted 1–2 min. When anaerobiosis was aimed for, the reaction mixture was incubated in the cuvette used for measurements for 7–10 min. Reduction with dithionite was performed by addition

of the solid sodium salt to the reaction mixture in the cuvette, followed by 1 min incubation.

Cuvettes (light path 2 mm) filled with reaction mixture were rapidly lowered into liquid nitrogen and kept here for at least 2 min before transfer to the Dewar flask in the spectrophotometer. The freezing time was about 15 s. As seen from the experiments presented, the reproducibility of freezing was very satisfactory.

Computations

Three types of spectra are dealt with in the present paper. Primary spectra are obtained directly from the spectrophotometer, i.e. they represent differences in absorbance of a treated system and a fully oxidized system. Difference spectra are calculated by the computer as the difference between two primary spectra. Derivative spectra are fourth-derivative spectra calculated by the method described by Butler & Hopkins (1970*a,b*). The derivative spectra indicate positions of maxima, but cannot be used as a quantitative measure of the absorbance in these maxima.

The reference channel in the dual-wavelength scannings served to correct for instrumental drift and differences in unspecific absorbance between the samples. A convenient wavelength is 570 nm. The reference wavelength has no relation to possible isosbestic points. Primary spectra were compared over the entire scanned range rather than by means of isosbestic points. Between the cytochrome *c* and cytochrome oxidase bands, no true isosbestic point exists, but the absorbance at 568 nm appear to be constant under many experimental conditions.

The computer was interfaced with an x - y recorder and the wavelengths of maxima read from the graphs of the derivative spectra (scale: 3 mm/nm). As the rounding-off error of the wavelength readings was rather large (about 0.1 nm), data could not be expected to fit the normal distribution. Identity of groups of wavelength measurements was tested with the non-parametric Mann-Whitney test, corrected for ties [see, for instance, Conover (1980)].

Results

The spectra presented here are all (except Fig. 3*b*) taken from an experimental series with one preparation of mitochondria in order to demonstrate the reproducibility of the techniques and of the intensification of the absorption bands at 77 K. Thus spectra have not been selected for, for instance, minimal noise. The three different types of spectra have been defined under 'Computations' in the Experimental section.

Fig. 2 shows the α -bands of three primary spectra. The unbroken line represents the spectrum obtained

Fig. 2. Cytochrome absorption spectra (α -bands)

The three primary spectra are: —, dithionite-reduced mitochondria; ---, mitochondria in State 4;, mitochondria in State 5. Substrates were succinate + glutamate (5.3 + 5.5 mM). Insert (with displaced wavelength scale), derivative spectrum of the State-5 spectrum. Absorption maxima are indicated by wavelengths at the derivative spectrum and also marked at the primary spectra. The protein concentration was 1.03 mg/ml.

Table 1. Absorption maxima (α -bands) of reduced cytochromes at 77 K

The wavelengths of the maxima were determined from derivative spectra obtained under a variety of experimental conditions. Values given are means $\pm$ s.d., with the number of independent determinations given in parentheses.

Cytochrome	Wavelength (nm)
c	548.27 $\pm$ 0.10 (186)
c ₁	553.26 $\pm$ 0.13 (167)
b(l)	556.99 $\pm$ 0.71 (223)
b(m)	560.61 $\pm$ 0.22 (173)
b(h)	564.50 $\pm$ 0.25 (194)

Table 2. Effect of experimental conditions on the wavelength of maximal absorption of reduced cytochrome b(l)

The experiments reported in Table 1 are grouped in five major groups according to treatment of the mitochondria. In 45 experiments, different treatments were used, e.g. combinations of inhibitors. Values given are means $\pm$ s.d., with the number of independent determinations in parentheses.

Treatment	Wavelength (nm)
Dithionite	556.08 $\pm$ 0.16 (17)
HQNO	556.33 $\pm$ 0.25 (52)
Anaerobiosis (State 5)	556.58 $\pm$ 0.29 (17)
Cyanide or TMPD + ascorbate	556.95 $\pm$ 0.31 (31)
Antimycin	557.83 $\pm$ 0.35 (61)

with dithionite (no further additions), whereas the spectra obtained with succinate + glutamate in the presence (State 4) and absence (State 5) of oxygen are indicated by the broken and dotted lines respectively. The State-5 reduction is very complete and there is no indication that cytochrome *b* is less reducible by substrates than the other cytochromes. A derivative spectrum (State 5) is inserted in Fig. 2. Four peaks are identified in the region between cytochrome *c* and cytochrome oxidase, namely cytochrome *c*₁ and three cytochrome *b* redox centres. The latter are named cytochromes *b(l)*, *b(m)* and *b(h)*. Wavelengths are not included in these designations since the maximum of at least one of the centres depends on the conditions.

Table 1 summarizes data for absorption maxima read from the derivative spectra. The data have been collected from a number of experimental series with different preparations of mitochondria that have been subjected to a variety of treatments, resulting in very different degrees of reduction of the cytochromes. Some of these treatments are indicated in Table 2. The derivative method is especially advantageous for detecting the maxima of cytochromes *c* and *c*₁, whereas cytochrome oxidase does not appear in this spectrum. The geometry of the maxima of the cytochrome *b* redox centres rather favours application of the derivative method, as evidenced by the precision of the determination of

cytochromes *b(m)* and *b(h)*. The maximum of cytochrome *b(l)* is apparently determined with much lower precision, although the primary spectra give no reason for expecting this. But subgrouping the data according to treatment of the mitochondria (Table 2) brings the s.d. values within the groups close to those for the cytochrome *b(m)* and *b(h)*. Further subgrouping can lower the s.d. values for some groups, but this is beyond the scope of the present paper.

The absorption maxima of the groups in Table 2 are all different from each other (significant at the $P = 0.001$ level). The statistical test does not exclude systematic errors in the application of the derivative method. Systematic errors caused by different heights of the neighbouring bands can, however, be excluded, because the grouped experiments in Table 2 are uncorrelated in this respect. In State 5, as well as in the presence of dithionite or TMPD + ascorbate, cytochromes *c* and *c₁* are highly reduced. With cyanide, different extents of redox level are established. Cytochrome *b* is reduced in the presence of HQNO or antimycin, but some of these experiments also involve reduction of cytochromes *c* and *c₁*, owing to the presence of cyanide or TMPD + ascorbate. Cytochrome *b(l)* therefore is considered a special kind of *b*-cytochrome, with absorption properties dependent on the way reduction is effected.

The State-5 maximum at 556.6 nm probably represents the least modified cytochrome *b(l)*, as it is independent of the nature of the substrates and the

energy level. Dithionite probably reacts directly with cytochrome *b(l)*, and this may explain the effect on the absorption maximum. TMPD + ascorbate is usually assumed to react at the level of cytochromes *c* or *c₁* (e.g. Lee *et al.*, 1967). The shift of the cytochrome *b(l)* maximum probably indicates a more direct interaction with Complex III. A similar effect has been suggested by Baum *et al.* (1967), who also report an effect of cyanide on Complex III. Antimycin and HQNO probably interfere directly with cytochrome *b(l)*, as will be described below.

Antimycin causes the largest shift of the cytochrome *b(l)* maximum (Table 2). Fig. 3(a) shows primary spectra of dithionite-reduced mitochondria in the presence and absence of antimycin. Under these conditions, antimycin shifts the maximum of cytochrome *b(l)* from 556.1 to 557.0 nm as determined by the derivative spectra. The difference spectrum shown below the primary spectra indicates that antimycin causes no other spectral changes and probably no change in absorption coefficients, as the negative peak almost balances the positive peak. A simple shift of the maximum of cytochrome *b(l)* explains the relation between the positions of the minimum and maximum in the difference spectrum and the maxima in the primary spectra. The hypsochromic effect of dithionite is also observed in the presence of antimycin.

In all experiments reported in the present paper (except in Fig. 3b), ethanol was evaporated from antimycin before addition of mitochondria. Ethanol

Fig. 3. Effects of antimycin and ethanol on the absorption of dithionite-reduced cytochromes

(a) —, control; ---, 1.9 μ M-antimycin added in absence of ethanol; insert: difference spectrum. All wavelengths marked are absorption maxima determined from the derivative spectra. The protein concentration was 1.03 mg/ml. (b) —, control; ···, 0.4% (v/v) ethanol added; ---, 3.1 μ M-antimycin added (ethanol concentration equal to 0.4%). The protein concentration was 0.94 mg/ml.

affects the reduced spectrum in the cytochrome c_1 region (Davis *et al.*, 1973) and in the cytochrome $b(h)$ region (Fig. 3b). The latter effect is apparently confined to intact mitochondria. A concentration of only 0.4% ethanol causes a shift of the maximum of cytochrome $b(h)$ to an extent that it can no longer be separated from the maximum of cytochrome $b(m)$ by the derivative method. The red shift due to antimycin and the blue shift due to ethanol result in a maximal increase of absorption at about 560 nm when antimycin is added in ethanol (Fig. 3b). This, in addition to the ignored existence of cytochrome $b(l)$, may explain why antimycin is often assumed to be bound to the cytochrome b redox centre absorbing at 562.5 nm at room temperature (cf. Slater, 1973). Ethanol and antimycin are not likely to change peak absorption coefficients, as the areas below the three spectra in Fig. 3b hardly differ from each other.

The properties of cytochrome $b(h)$ appear very sensitive to factors that affect the mitochondrial membrane. Dimethyl sulphoxide and Triton X-100 cause effects similar to the effect of ethanol, i.e. increase in the absorption due to cytochrome c_1 and a blue shift of the absorption maximum due to cytochrome $b(h)$.

Pigeon heart mitochondria prepared as described above exhibit negligible endogenous respiration, but partial reduction of cytochrome b occurs in the presence of antimycin or HQNO. As shown in Fig. 4, only cytochromes $b(l)$ and $b(h)$ are reduced in the presence of antimycin, whereas HQNO causes reduction of only cytochromes $b(l)$ and $b(m)$. Incubation of mitochondria in the presence of both inhibitors results in spectra that are qualitatively combinations of those obtained when the inhibitors are present alone and consequently show less reduction of cytochromes $b(m)$ and $b(h)$. With both inhibitors present, the maximum of the primary spectrum is displaced from the maximum of cytochrome $b(l)$, as when HQNO is added alone. Hence some cytochrome $b(m)$ is reduced in both cases. The position of the cytochrome $b(l)$ maximum with both inhibitors added simultaneously and in optimal amounts is 556.86 ± 0.24 nm (mean $\pm$ s.d., $n = 19$). This position may correspond to 35% of the antimycin-affected cytochrome $b(l)$ (maximum at 557.83 nm) and 65% of the HQNO-affected cytochrome $b(l)$ (maximum at 556.33 nm, see Table 2). The data are in favour of a site binding either antimycin or HQNO. It is likely that the quantitatively different effects of the two inhibitors added simultaneously

Fig. 4. Reduction of cytochromes by endogeneous substrates: effects of antimycin and HQNO

Primary spectra are shown on the left and derivative spectra on the right. The absorption maxima determined from derivative spectra are marked at the primary spectra. Experiments were performed as described in the Experimental section ([antimycin] = $1.9 \mu\text{M}$; [HQNO] = $23.8 \mu\text{M}$; [protein] = 1.03 mg/ml).

is due to different binding kinetics rather than to different binding equilibria, because, as will be described below, neither is able to displace the other once it is bound.

The reduction of cytochromes by succinate + glutamate in the presence of antimycin or HQNO is shown in Figs. 5 and 6 respectively. The spectra of the system completely reduced by further addition

Fig. 5. Reduction of cytochromes by succinate + glutamate in the presence of antimycin

The two primary spectra, the difference spectrum, and the three derivative spectra are marked according to experimental conditions: A, succinate + glutamate (0.35 + 0.37 mM) with 1.9 μ M-antimycin; B, as A, but reduced with dithionite. The arrows indicate peak values as determined from the derivative spectra. The protein concentration was 1.03 mg/ml.

Fig. 6. Reduction of cytochromes by succinate + glutamate in the presence of HQNO

The two primary spectra, the difference spectrum, and the three derivative spectra are marked according to experimental conditions: A, succinate + glutamate (0.35 + 0.37 mM) with 23.8 μ M-HQNO; B, as A, but reduced with dithionite. The arrows indicate peak values as determined from the derivative spectra. The protein concentration was 1.03 mg/ml.

of dithionite are also shown, as well as the difference spectra in the intervals of interest. Derivative spectra are included in the Figures. In the presence of added substrates, the inhibitor effects may be expected to appear more complex, since leakage through inhibited paths and possible ATP formation may affect the pattern of reduction. The leakage is detectable as a small rate of respiration in the presence of inhibitors and as the small reduction of cytochrome *c* (Figs. 5 and 6).

From the results with endogenous substrates it may be expected that cytochromes *b(l)* and *b(h)* are reduced, and cytochrome *b(m)* remains oxidized, in the presence of antimycin. The difference spectrum of Fig. 5 shows almost complete reduction of cytochrome *b(l)* in the presence of substrates and antimycin. The small unreduced part of cytochrome *b(l)* is apparently not affected by antimycin, as seen from its absorption maximum at 556.6 nm. Very little, if any, cytochrome *b(m)* is reduced in the presence of substrates and antimycin. Identification of this maximum from the derivative spectrum is hardly possible (curve A, Fig. 5). Cytochrome *b(h)* is reduced as expected, but the reduction is not complete in the presence of substrates and antimycin. The degree of reduction of cytochrome *b(h)* relative to cytochrome *b(l)* appears to be the same as in the experiments of Fig. 4.

In the presence of HQNO (Fig. 6), succinate + glutamate cause complete reduction of cytochromes *b(l)* and *b(m)*, as expected from the experiments of Fig. 4. The peaks are hardly detectable in the derivative of the difference spectrum. Some cytochrome *b(h)* is, however, reduced, probably owing to the leakage mentioned above.

The spectrum with HQNO added after antimycin is identical with the spectrum with antimycin alone (curve A, Fig. 5), and the spectrum with antimycin added after HQNO is identical with the spectrum with HQNO alone (curve A, Fig. 6). Thus the two inhibitors are probably unable to displace each other from a common binding site.

Discussion

The metabolic activity of cytochrome *b* appears to be very sensitive to the integrity of the mitochondrial preparation, much more sensitive than, for instance, the respiratory-control phenomenon. In the mitochondrial preparation used in the present study the entire pool of *b*-cytochromes is reduced by substrates in the absence of oxygen (State 5). Mitochondria with normal rate of ADP phosphorylation but only partial reduction of the cytochrome *b* redox centres in State 5 may be obtained by slight modifications of the preparation procedure. We believe that much variability in the findings with respect

to cytochrome *b* may be ascribed to differences in integrity of the preparations used.

The three cytochrome *b* redox centres described in the present paper with absorption maxima at 556.6 (State 5), 560.6 and 564.5 nm are probably identical with cytochromes *b*-558, *b*-562 and *b*-566 [absorption maxima at room temperature, see Wikström (1973)]. In support of this, the wavelength shift due to freezing at 77 K is identical for all centres (1.5 nm). We prefer, however, the names cytochromes *b(l)*, *b(m)* and *b(h)*, because the best spectral resolution is obtained at low temperature and the maximum of at least one of the centres depends on the conditions of measurement. Cytochrome *b*-558 is often assumed to be a shoulder on the cytochrome *b*-566 band (e.g. Sato *et al.*, 1971). Yu *et al.* (1972) and Higuti *et al.* (1975) favour the assumption that cytochrome *b*-558 is a separate centre, whereas Malviya *et al.* (1980) maintain that cytochrome *b*-558 is a shoulder. As shown in the present paper, cytochrome *b(l)* may be reduced without any reduction of cytochrome *b(h)* (Fig. 4). The assumption that cytochrome *b(l)* is a shoulder on the cytochrome *b(h)* band is thereby ruled out.

Reliable quantification of the individual cytochrome *b* redox centres is not yet possible, but some information about the relative amounts may be gained from comparison of a number of spectra. It is most likely that cytochrome *b(l)* is the major component in intact pigeon heart mitochondria. This is in obvious contrast with data for rat liver mitochondria, where cytochrome *b*-562 was reported to be the major component (Wikström, 1971). In submitochondrial particles, two cytochromes *b*-562 (or *b_K*) have been reported (Berden & Oppenheims, 1972). They differ with respect to redox potentials and it has been suggested that the high-potential compound is a preparation artefact (Wikström, 1973). Unspecific alteration of the mitochondrial membrane (e.g. by ethanol) lowers the maximum wavelength of cytochrome *b(h)* to the range of cytochrome *b(m)* (Fig. 3b). Isolated cytochrome *b* haemoprotein has absorption properties very similar to those of cytochrome *b(m)* (Weiss, 1976; von Jagow *et al.*, 1978). It may be considered whether or not cytochrome *b(m)* owes its existence to conversions of the other cytochrome *b* redox centres, but so far there is no conclusive experimental evidence against assuming that cytochrome *b(m)* is a native member of the respiratory chain.

The maximum wavelength of cytochrome *b(h)* is high compared with the value for the comparable redox centre reported by Davis *et al.* (1973) (564.5 versus 562.5 nm). Those authors, however, used preparations of ox heart origin, excluding intact mitochondria, and they did not observe the shift of absorption maximum due to ethanol. Cytochrome *b_T* as defined by Chance and co-workers (e.g. Sato

et al., 1971) absorbs maximally at 562.5 nm (77 K). This cytochrome is, however, not identical with cytochrome *b(h)*, as it probably is not a single redox centre (Wikström, 1973).

In the range 556–557 nm only one absorption maximum is detected by the derivative method, and this maximum, ascribed to cytochrome *b(l)*, can account for almost all absorbance. Therefore cytochrome *b(l)* is presumably only one component, in spite of the shifts of absorption maximum. These shifts are caused by compounds that probably all interfere with Complex III. The shifts cannot, however, be taken as evidence for a direct interaction with cytochrome *b(l)*. Antimycin causes the largest shift of the cytochrome *b(l)* peak and the shift is correlated with the degree of inhibition (results not shown). Antimycin is known to be bound in amounts stoichiometric with cytochrome *c₁* (Pumphrey, 1962; Rieske *et al.*, 1967). Several mechanisms of action have been proposed. These involve binding to a special polypeptide (Bell & Capaldi, 1976), to an iron-sulphur protein (Tappel, 1963), to a compound (X) (Baum *et al.*, 1967), to cytochrome *b_T* (von Jagow & Klingenberg, 1972), and to cytochrome *b-562* [for a review, see Slater (1973)]. Furthermore, binding in a cavity in Complex III has been suggested by Rieske (1976). Our data indicate that antimycin probably is bound in the vicinity of, or directly to, cytochrome *b(l)*, as this is highly reduced in the presence of antimycin and furthermore is the only cytochrome *b* redox centre with shifted maximum.

The competition of HQNO and antimycin for the same site is here evidenced by spectrometric observations. The assumption is in agreement with that of van Ark & Berden (1977), who measured HQNO fluorescence in the supernatant and found equal number of binding sites for the two inhibitors. Nijs (1967) made the same suggestion on the basis of the mutual effects of the two inhibitors on respiratory rates. van Ark & Berden (1977), using sub-mitochondrial particles, observed that antimycin was able to displace HQNO already bound, but not vice versa. Our data, obtained with intact mitochondria, indicate that neither inhibitor was able to displace the other once it was bound.

In the presence of antimycin, cytochromes *b(l)* and *b(h)* were reduced, and in the presence of HQNO, cytochromes *b(l)* and *b(m)* were reduced. Supposing that inhibition does not introduce new pathways, these observations are incompatible with the assumption of a linear chain between the three cytochrome *b* redox centres. A minimal requirement of a model for this system is that it must explain that the small inhibitor molecules, apparently reacting at the same binding site, cause different effects with respect to the redox centres. The inhibitors may act by affecting the conformation of Complex

III (cf. Rieske, 1976), but proposition of a more detailed mechanism obviously must await further investigation.

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