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ENERGY-DEPENDENT REDOX REACTIONS OF TWO *b*-TYPE CYTOCHROMES IN BROWN ADIPOSE TISSUE MITOCHONDRIA

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SUMMARY

1. Brown adipose tissue mitochondria from guinea pigs reveal kinetically three cytochrome *b* species, two of which have indistinguishable absorption spectra at room temperature, *i.e.* the α-band at 561.5 nm. The third species is a twin hemochromogen with the α-bands at 565 nm and 558 nm. In cyanide-inhibited mitochondria a certain species of b_{561} is slowly reduced by ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) whereas the other species of b_{561} is rapidly reduced by succinate in the coupled and uncoupled state.

2. In the presence of succinate only $b_{565/558}$ is reduced by energization of the mitochondria by ATP, whereas in the absence of this substrate both species are reduced in reversed electron flow from ascorbate/TMPD. The energy-dependent reductions of the two cytochromes, which occur in rougly equimolar amounts, were strongly pH dependent and was found to have different K'_m values for ATP, *i.e.* 120 μM and 240 μM for b_{561} and $b_{565/558}$, respectively.

3. Antimycin A induces a reduction of $b_{565/558}$ in the forward electron flow (from succinate), with a typical sigmoidal titration curve. On the other hand, when antimycin A inhibits the reversed electron flow (from ascorbate/TMPD) supported by added ATP, the titration curve was not sigmoidal. Thus, at a critical concentration of the inhibitor the response to added ATP switched from 20% reduction to 8% oxidation. At this antimycin A concentration cytochrome b_{561} is selectively oxidized by ATP in the presence of succinate. This oxidation suggests that under these conditions cytochrome b_{561} equilibrates neither with $b_{565/558}$ nor with c_1, but with ubiquinone and that b_{561} lowers its redox potential upon energization of the mitochondria.

4. The present study has shown that the oxidation–reduction of both species of cytochrome *b* are energy linked and not only $b_{565/558}$ (b_T) as previously proposed [Chance, B., Wilson, D. F., Dutton, P. L. and Erecinska, M. (1970) *Proc. Natl. Acad. Sci. U.S.* 66, 1175–1182]. Thus, both cytochromes *b* may participate in energy transduction at coupling Site 2.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

INTRODUCTION

Although physicochemical studies on purified cytochrome *b* of the mammalian respiratory chain indicated the presence of only one chemical species[1], recent studies on intact mitochondria have clearly revealed the presence of more than one species of cytochrome *b* which are operable potentiometrically[2–4], kinetically[3] as well as spectrally[5,6]. Thus, in 1970 Wilson and Dutton[2] reported that rat-liver mitochondria contain two cytochrome *b* species, differing in mid-point potential by 90 mV (pH 7.3), and that addition of ATP increases the potential of the lower-potential species from −55 mV to +245 mV. Similar results have been reported in pigeon heart mitochondria[3] as well as in bovine heart mitochondria[4]. Furthermore, evidence has been obtained by Chance *et al.*[3] for the existence of two kinetically distinct species in pigeon heart mitochondria which have been found to have different spectral properties[5,6]. These cytochromes *b* have been designated[3] b_K (b_{Keilin}) and b_T ($b_{Transducing}$). Cytochrome b_K revealed at room temperature a single symmetrical α-band at 561 nm and was readily reduced by succinate in both coupled and uncoupled mitochondria[6]. Cytochrome b_T on the other hand, revealed a double α-band at 565 nm and 558 nm and was readily reduced by succinate in the presence of ATP but not in uncoupled mitochondria[6]. Thus, according to Chance *et al.*[3] cytochrome b_K has a redox potential which is independent of the energy state whereas that of cytochrome b_T increases from a low potential to a high potential form upon energization of the mitochondria by ATP. On the other hand, Berden *et al.*[7] have recently presented evidence that both cytochrome *b* species of bovine heart mitochondria change the apparent mid-point potential on adding ATP.

This discrepancy between conclusions based on potentiometric and spectral studies[3,7] is the main problem raised in the present study. Our spectral and kinetic studies on brown adipose tissue mitochondria have clearly demonstrated that both cytochrome b_{561} and cytochrome $b_{565/558}$ are reduced by reversed electron flow upon energization of the mitochondrial inner membrane by ATP in the absence of added succinate. Furthermore, evidence is obtained that cytochrome b_{561} also changes its redox potential upon energization and not only $b_{565/558}$. A preliminary account of this work has already appeared[8].

MATERIALS AND METHODS

Animals and preparation of mitochondria

3–4-week-old guinea pigs (Pir/Srr/c stain) were cold exposed at an environment of 5 °C for at least 6 days before sacrifice. Mitochondria from the interscapular brown adipose tissue were prepared and the protein determined as described[9]. Reduced *minus* oxidized difference spectra revealed that erythrocytes (hemoglobin) had been completely removed.

Chemicals

Antimycin A, rotenone and ATP were obtained from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) was a gift from Dr P. G. Heytler of Du Pont, Wilm., U.S.A. Other chemicals were of the highest purity commercially available.

Incubation of mitochondria

The mitochondria were incubated at 25 °C in a medium containing in a volume of 1 ml: 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 6.8, unless otherwise stated; 5 mM potassium phosphate buffer, pH 6.8; 135 mM sucrose; 2 mM EDTA; 1 mM $MgCl_2$; 3 μM rotenone; and 3.3 mM KCN. The concentration of ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) were 4.2 mM and 90 μM, respectively. All acids were pH adjusted by KOH. Other additions are indicated in legends to figures.

Spectrophotometry

The measurements of oxidation–reduction level of *b*-type cytochromes were performed in cuvettes of 10-mm light path using an Aminco-Chance dual-wavelength spectrophotometer with both monochromators calibrated with reduced cytochrome *c* at an accuracy of ± 0.2 nm (for wavelength setting, see Results and legends to figures). The temperature was thermostatically controlled at 25 °C.

Difference spectra were also measured at 25 °C in a Shimadzu recording spectrophotometer (Model MPS-50L) calibrated against several emission bands of a mercury arc. Cuvettes of 10-mm light path were used.

RESULTS

The spectral properties of the b-type cytochromes

To the suspension of brown adipose tissue mitochondria was added KCN to block the terminal part of the respiratory chain as well as rotenone to block the utilization of endogenous NADH-linked substrates. Furthermore, ascorbate and TMPD were added to reduce the cytochromes c_1, *c* and aa_3 and to block the ATP responses in this part of the chain[10–12]. From Figs 1A–1C it is seen that a slow absorbance increase follows from the addition of ascorbate/TMPD. The species reduced has been identified as a *b*-type cytochrome with maximum around 561.5 nm, *i.e.* b_{561}[9]. This 'background' reduction by ascorbate/TMPD did not disappear upon the subsequent additions, *e.g.* of succinate and ATP, and should be considered in all progress curves shown in Figs 1A–1C. Thus, this part of cytochrome b_{561} is probably not involved in the functional respiratory chain.

As expected, the addition of succinate to ascorbate/TMPD-supplemented mitochondria (Fig. 1A) also resulted in reduction of a cytochrome *b* species with an absorption maximum around 561.5 nm (Fig. 2A, upper curve). The reduction induced by the subsequent addition of ATP (Fig. 1A) resulted in an asymmetric cytochrome *b* spectrum (Fig. 2A, lower curve). These spectra were resolved by an electronic curve resolver (Hirschberg, H. and Flatmark, T., unpublished) into two Gaussian curves with their maxima at 565 nm and 558.5 nm, respectively. By calculation of the area under the three resolved curves (Fig. 2A) the stoichiometry of $b_{561}:b_{565}:b_{558}$ was found to be 5.02:5.0:0.98.

When ATP was added to the mitochondria after ascorbate/TMPD (Fig. 1B) the absorbance difference between 564 and 575 nm showed a significantly higher steady-state increase than expected from Fig. 1A. Furthermore, a spectrum with its maximum at about 563 nm was obtained (Fig. 2B), *i.e.* almost exactly the summation spectrum of b_{561} and $b_{565/558}$ (Fig. 2A). This result together with the

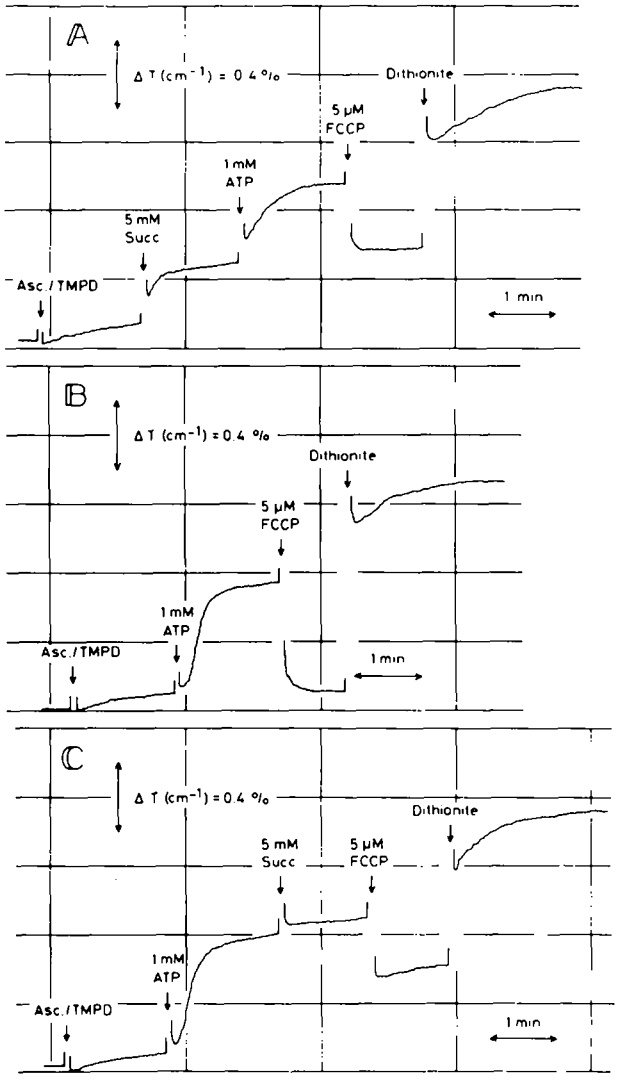


Fig. 1. (A) The reduction of *b*-type cytochromes in brown adipose tissue mitochondria by ascorbate/TMPD, succinate and ATP, and the effect of FCCP. The mitochondria (pooled sample of 3 animals) were suspended in the standard incubation medium (see Methods) at 0.42 mg of protein per ml, pH 6.8. The change in transmission ΔT (%)$=\Delta(T_{564\text{ nm}-575\text{ nm}})$. (B) The reduction of *b*-type cytochromes induced by ATP in the absence of succinate and the effect of FCCP. Experimental details as in A. (C) The reduction of *b*-type cytochromes by ATP and succinate and the effect of FCCP. Experimental details as in A.

fact that the total reduction induced by ATP is sensitive to the uncoupler FCCP (Fig. 1B) indicate that in the absence of succinate the redox state of b_{561} as well as of $b_{565/558}$ is dependent on the mitochondrial energy state. This conclusion is also supported by the observation that the extent of the subsequent reduction by succinate was considerably reduced (Fig. 1C) as compared to that observed when

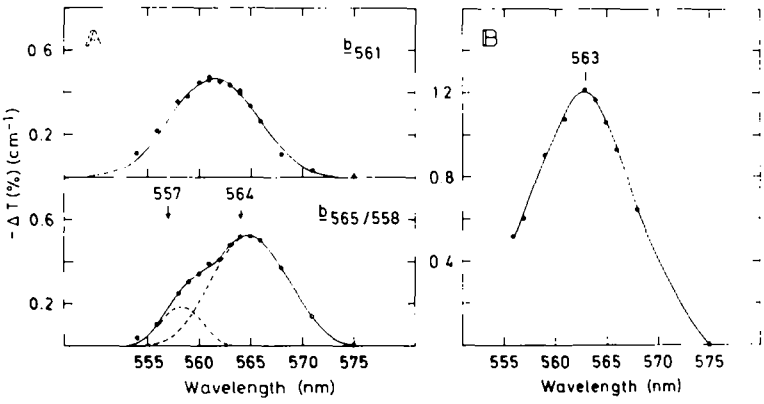
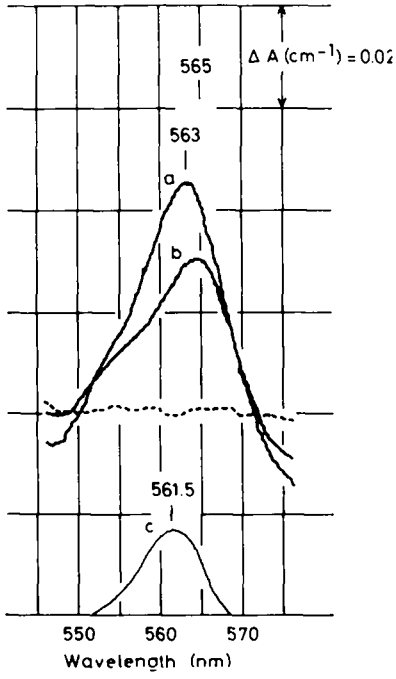


Fig. 2. (A) The absorption spectra of cytochrome b_{561} reduced by succinate (upper trace) and cytochrome $b_{565/558}$ reduced by ATP added after succinate (lower trace). The mitochondria were suspended in the standard incubation medium (see Methods) at 0.65 mg of protein per ml. Succinate and ATP were added in sequence as shown in Fig. 1A and the steady-state change in transmission [ΔT (%)] was recorded with the reference wavelength set at 575 nm and the measuring wavelength as indicated on the abscissa. The experimental values fitted in the upper trace a single Gaussian curve (——) and in the lower trace a summation curve (· —·) and two Gaussian curves (- - - -). The arrows indicate the wavelengths used (*i.e.* 557 nm and 564 nm) for a more selective study of the effect of pH and ATP concentration on the 558-nm and 565-nm transitions. (B) The absorption spectrum of cytochromes *b* reduced by ATP after ascorbate/TMPD. The mitochondria were suspended in the standard incubation medium (see Methods) at 0.87 mg of protein per ml. ATP and FCCP were added as shown in Fig. 1B and the spectrum was obtained as described above.



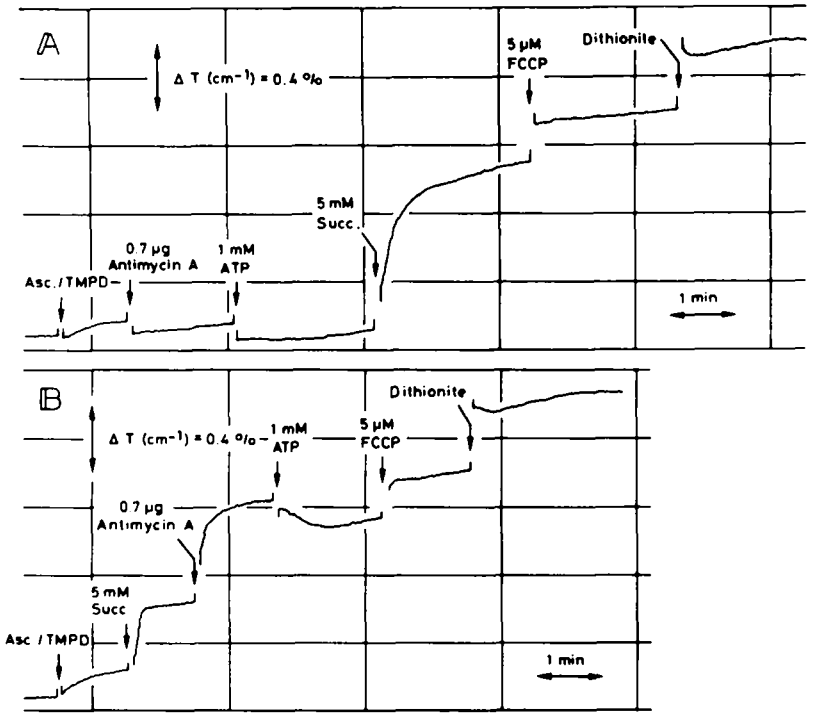


Fig. 4. Inhibition by antimycin A of the ATP-induced reduction of cytochromes *b* (A) and the effect of ATP on the redox level of cytochromes *b* reduced by succinate in the presence of antimycin A (B). The mitochondria were suspended in the standard incubation medium (see Methods) at 0.63 mg of protein per ml; pH 6.8. The change in transmission $\Delta T\,(\%) = \Delta\,(T_{564\text{ nm}} - T_{575\text{ nm}})$.

this substrate was added before ATP (Fig. 1A). Under such conditions, *i.e.* in the presence of succinate (Figs 1A and 1C), the response to FCCP is considerably smaller than in the absence of substrate (Fig. 1B) in accordance with the fact that succinate maintains cytochrome b_{561} reduced in both the coupled and uncoupled state[5,6].

In order to identify the absorption bands of reduced cytochromes *b* induced by ATP in the absence of added succinate, difference spectra were recorded as described in Fig. 3. It is clearly seen that the additional reduction obtained when ATP is added in the absence of succinate (Figs 1B and 1C) as compared to that obtained in the presence of this substrate is due to reduction of cytochrome b_{561}.

Fig. 3. Reduced *minus* oxidized difference spectra of cytochromes *b* in brown adipose tissue mitochondria. Curve a, ascorbate/TMPD was added to both the reference and the sample cuvette followed by 2 mM ATP to the sample. The spectrum was recorded at steady-state level. Curve b, ascorbate/TMPD + 5 mM succinate were added to both cuvettes followed by 2 mM ATP to the sample and the spectrum was recorded at steady-state level. Curve c, the calculated difference spectrum between a and b. The mitochondria were suspended in the standard incubation medium (see Methods) at 3.9 mg of protein per ml.

Thus, under these experimental conditions both cytochromes ($b_{561}+b_{565/558}$) are reduced upon energization of the mitochondrial inner membrane by ATP.

Effect of antimycin A on the redox reactions of b-type cytochromes

The reduction of the *b*-type cytochromes by ATP in the absence of succinate described above is induced by reversed electron flow from ascorbate/TMPD *via* cytochromes *c* and c_1 since it is completely inhibited by antimycin A at a concentration $\gtrsim$0.25 μg per mg of protein (Fig. 4A). It should be noted that this inhibition revealed an interesting titration curve (Fig. 5A) not previously observed. Thus, at a critical concentration of the inhibitor the response to added ATP switched from 20% reduction to 8% oxidation.

On the other hand, by adding antimycin A after succinate (Fig. 4B) a reduction of cytochrome $b_{565/558}$, similar to that induced by ATP, was observed. The summation spectrum thus obtained had its maximum at about 563.5 nm, *i.e.* shifted by approx. 0.5 nm towards the red as compared to the spectrum obtained by succinate *plus* ATP[13]. However, in contrast to the ATP-induced reduction of cytochrome $b_{565/558}$, that induced by antimycin A was completely independent of pH in the range tested (Fig. 6, ●—●) and insensitive to the uncoupler FCCP (curve not shown). Fig. 5B shows the reducibility of cytochrome $b_{565/558}$ by succinate in the presence of

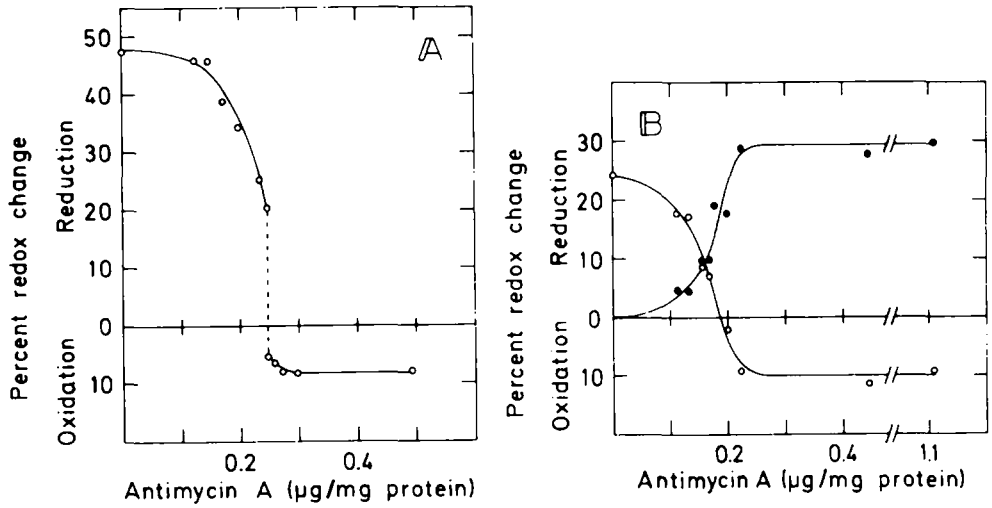


Fig. 5. (A) Effect of antimycin A concentration on the ATP-induced redox changes of cytochromes *b*. The mitochondria were suspended in the standard incubation medium (see Methods) at 0.41 mg of protein per ml and preincubated in the presence of antimycin A. 1 mM ATP was added as shown in Fig. 4A and the redox level measured at steady state. (B) Effect of antimycin A concentration on the reducibility of cytochrome $b_{565/558}$ by successive additions of succinate (●) and ATP (○). The mitochondria were suspended in the standard incubation medium (see Methods) at 0.45 mg of protein per ml; pH 6.8. The experiments were conducted according to Fig. 4B. After 5 mM succinate, antimycin A was added in concentrations as shown and the steady-state level recorded (●). 1 mM ATP was then added and the new steady-state measured (○). 100% reduction represents the difference in transmission Δ ($T_{564\ nm}-T_{575\ nm}$) between the level obtained by dithionite and the initial ascorbate/TMPD level. The reduction induced by the initial succinate addition (in B) corresponded to 15%.

increasing amounts of antimycin A. In this case the titration curve revealed the typical sigmoidal form (for review, see ref. 14). The further reduction of cytochrome $b_{565/558}$ by ATP decreased inversely with its increased reducibility by antimycin A.

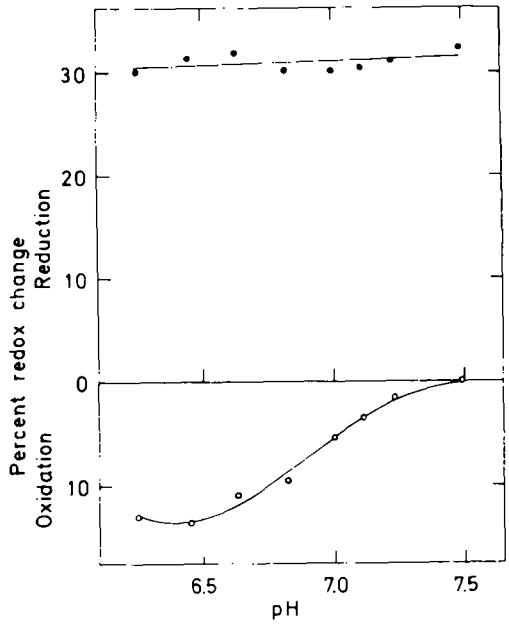


Fig. 6. Effect of pH on the reduction of cytochrome $b_{565/558}$ by succinate in the presence of antimycin A (●) and on the ATP-induced oxidation of cytochrome b_{561} in the presence of succinate and antimycin A (○). The mitochondria were suspended in the standard incubation medium (see Methods) at 0.44 mg of protein per ml. The experiments were performed as described in Fig. 4B. 100% reduction/oxidation as defined in the legend to Fig. 5.

Addition of ATP after succinate at saturating concentrations of antimycin A resulted in a partial oxidation of the cytochrome *b* complex (Fig. 4B) which was strongly pH dependent (Fig. 6, ○—○). This oxidation was reversed by FCCP (Fig. 4B) and completely inhibited by oligomycin (figure not shown) and thus energy dependent. Spectral analysis of the component oxidized revealed an absorption maximum at 561.5 nm (Fig. 7).

Effect of ATP concentration

At saturating concentrations of ATP the percentage reduction at pH 6.8 in the absence and presence of succinate was 58% ($b_{561}+b_{565/558}$ and 28% ($b_{565/558}$), respectively (for further details, see Fig. 6A of ref. 9). From the ATP titration curves (Fig. 6A, ref. 9) it was further calculated that the ATP concentration needed for half maximal reduction decreased from 240 μM in the presence of succinate to 120 μM when the reaction was studied without prior addition of this substrate.

Effect of pH on the succinate and ATP induced reduction of b-type cytochromes

From Fig. 8B it is seen that the reduction of b_{561} by succinate added before ATP increased by increasing pH. The subsequent reduction of $b_{565/558}$ induced

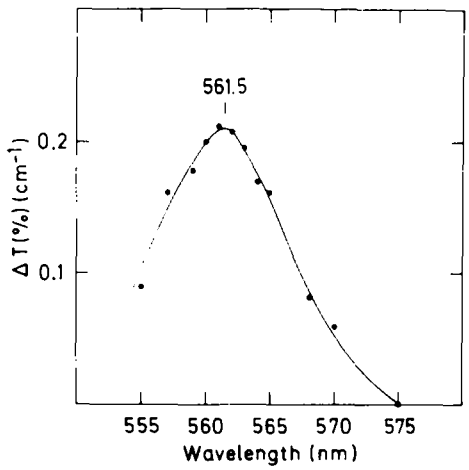


Fig. 7. The absorption spectrum of the cytochrome *b* species oxidized by ATP following reduction of the cytochrome *b* complex by succinate in the presence of antimycin A. The mitochondria were suspended in the standard incubation medium, pH 6.8 (see Methods) at 0.54 mg of protein per ml. After maximal reduction by 5 mM succinate in the presence of antimycin A (0.5 μg/ml), 1 mM ATP was added followed by 5 μM FCCP as shown in Fig. 4B. The steady-state change in transmission [$\Delta T(\%)$] induced by ATP was recorded with the reference wavelength set at 575 nm and the measuring wavelength as shown.

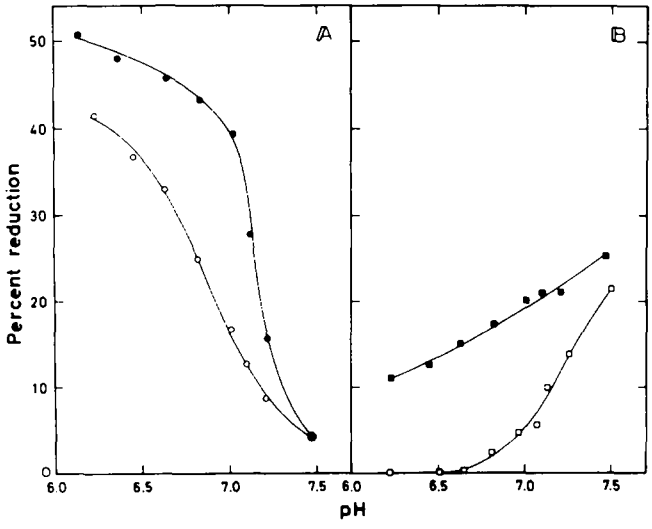


Fig. 8. (A) Effect of pH on the reduction of cytochromes *b* (●) induced by 1 mM ATP and on the reduction of cytochrome $b_{565/558}$ (○) induced by 1 mM ATP when added after 5 mM succinate. The experiments were conducted corresponding to Figs 1A and 1B and appropriate controls were taken to make corrections for the slow decrease in the ATP response by time. 0.49 mg of mitochondrial protein per ml (pooled sample of 2 animals). (B) Effect of pH on the reduction of cytochrome b_{561} induced by 5 mM succinate when added before (■) and after (□) ATP. The experiments were conducted corresponding to Figs 1A and 1C. The concentrations of mitochondrial protein were 0.49 mg per ml (■) and 0.47 mg per ml (□). 100% reduction as defined in the legend to Fig. 5.

by ATP was even more pH dependent but with an apparent optimum around 6.0 (41% reduction at pH 6.2) and only a minimal reduction above pH 7.5 (Fig. 8A). This difference in pH dependency of the substrate and ATP-induced reductions were even more pronounced when ATP was added prior to succinate (Figs 8A and 8B). In this case the ATP-induced reduction of $b_{565/558}+b_{561}$ also revealed an apparent pH optimum at around pH 6.0. Furthermore, it can be seen from Fig. 8B that after energization of the mitochondria by ATP less cytochrome b_{561} is available for reduction by succinate. Above pH 6.6, the extent of cytochrome b_{561} reduction by succinate increases almost inversely with the ATP-induced reduction (as studied at 564 nm).

Cytochrome $b_{565/558}$ — one or two species?

It is still uncertain whether the spectrum of cytochrome $b_{565/558}$ represents a twin hemochromogen[6] or is due to two different cytochrome *b* species[15,16]. In order to get more information on this problem we have studied the effect of ATP concentration as well as the effect of pH on the ATP-induced reduction of cytochrome $b_{565/558}$ in the presence of succinate. In brown adipose tissue mitochondria the 565-nm transition could be followed selectively at, *e.g.* 564 nm, whereas the 558.5-nm transition could be followed almost selectively at 557 nm (see Fig. 2A). From Fig. 9 it is seen that the same dependence on ATP concentration (A) and pH (B) was observed independently of the wavelength pair selected.

DISCUSSION

Brown adipose tissue mitochondria offer particular advantages for the study of energy-linked redox reactions, *e. g.* of the cytochrome *b* complex, for two reasons. First, they have a very high content of respiratory chain components[17], and secondly, when freshly isolated these mitochondria are completely deenergized by a physiological mechanism[9,18]. Thus, under the experimental conditions used in the present study the cytochrome *b* complex is almost completely in the oxidized form and the reductions induced by added ATP represent the total energy dependent redox reactions of these mitochondria in contrast to the situation, *e.g.* in liver mitochondria where a significant and not identified part of the cytochrome *b* complex is already reduced by the endogenous ATP[9].

Our studies (Figs 1A and 1C and Fig. 2A) have revealed the same spectral species of cytochrome *b* as described in pigeon heart[3,5,6] and rat liver mitochondria[16] and that cytochrome b_{561} is reduced by succinate in both the coupled and uncoupled state. Therefore, in order to observe an effect of ATP on this species, the nucleotide must be added in the absence of succinate. Thus, in the presence of ascorbate/TMPD both cytochrome b_{561} and cytochrome $b_{565/558}$ are stoichiometrically reduced by ATP (Figs 2B and 3) in strongly pH dependent reactions (Fig. 8A). These reductions which have not previously been observed in other types of mitochondria, proceed by reversed electron flow from ascorbate/TMPD *via* cytochromes $c+c_1$ as shown by their complete inhibition by antimycin A (Fig. 4A). Furthermore, after reduction of both cytochromes *b* by succinate has been obtained in the presence of antimycin A, cytochrome b_{561} is still influenced by the 'energy potential' as shown by its oxidation upon energization of the mitochondria by ATP (Figs 4B and 6), in

accordance with similar findings with rat liver[19] and pigeon heart mitochondria[20]. Except for ubiquinone, all the components of the respiratory chain, with which cytochrome b_{561} may equilibrate, are fully reduced under these conditions, and the explanation for this reaction must be that the redox potential of cytochrome b_{561} is lowered by ATP. This conclusion is further supported by the fact that the extent of ATP-induced oxidation in the presence of 10 mM succinate *plus* 4.5 μM palmityl-L-carnitine (in the absence of rotenone) was 15.5% as compared to 7.1% in the presence of 0.5 mM succinate. Thus, when the steady-state reduction of ubiquinone is increased, the energy-dependent oxidation of cytochrome b_{561} increases. That this cytochrome *b* species may be energetically reduced or oxidized depending on the experimental conditions, is in agreement with Berden *et al.*[7] that not only cytochrome $b_{565/558}$ changes its apparent mid-point potential on adding ATP, but that part of cytochrome b_{561} increases and part of it decreases its mid-point potential in the presence of the nucleotide. Attempts to determine the redox potentials of the *b*-type cytochromes of brown adipose tissues mitochondria in the presence of ATP by the method of Wilson and Dutton[2] were, however, unsuccessful due to lability of the mitochondria and too slow equilibration of the system.

According to the present findings the redox levels of both cytochromes *b* are in equilibrium with the mitochondrial 'energy potential'. From the difference between the ATP titration curves (Fig. 6A of ref. 9) it can be calculated that the ATP concentration needed for half-maximal reduction of cytochrome b_{561} is 120 μM. In spite of partly charged particles by succinate, 240 μM ATP was needed for half-maximal reduction of cytochrome $b_{565/558}$. This difference may indicate that cytochrome b_{561} operates at a lower level of the 'energy potential'.

The energy-dependent oxidation of cytochrome b_{561} in the presence of antimycin A shows that the inhibitor probably does not affect the energy transfer between cytochrome b_1 and X~I as depicted in the model of Wilson *et al.*[21], but rather that the effect is directly on one of the cytochrome *b* species, probably $b_{565/558}$. This conclusion is also supported by the antimycin A titration curve (Fig. 5A) which suggests that at a critical concentration of the inhibitor, where it is bound in stoichiometric amount to cytochrome $b_{565/558}$, the equilibrium between b_{561} and $b_{565/558}$ is abolished. Previous titrations have in fact shown that antimycin A inhibition is complete when approx. one molecule of the inhibitor is bound per two molecules of cytochrome *b*[22,23].

As already demonstrated in intact rat heart mitochondria[24] and in submitochondrial particles[23,25] the curve describing the effect of different concentrations of antimycin A on the reduction of cytochrome $b_{565/558}$ in forward electron flow (from succinate) is sigmoidal (Fig. 5B). However, in contrast to the findings with submitochondrial particles from bovine heart[24] the shape of the antimycin A titration curves with intact brown adipose tissue mitochondria is different in forward (Fig. 5B). and reversed electron flow (from ascorbate/TMPD supported by added ATP, Fig. 5A). This experimental fact stresses the problem of extrapolating from studies with submitochondrial particles to the situation in intact mitochondria as recently discussed in detail by Norling *et al.*[26].

The very strong pH dependencies of the energy-linked reactions in brown adipose tissue mitochondria may partly be explained by the pH dependency of the

redox potential of the *b*-type cytochromes[27,28]. The difference in pH dependency, however, between the ATP- and substrate-induced redox changes and the lack of effect of pH on the antimycin A-induced reduction probably more reflect the importance of the intramitochondrial proton activity for the generation of the 'energy potential'[29]. As previously discussed[9,17,18] these pH–activity curves seem to be specific for loosely coupled brown adipose tissue mitochondria and related to their thermogenic function since they are not observed in liver mitochondria[9]. This difference between the two types of mitochondria may be more related to a difference in proton permeability than to fundamental differences in their mechanism of energy conservation.

It is not yet clear whether or not the spectrum of cytochrome $b_{565/558}$ represents one or two species of cytochrome *b*. Based on different pH dependencies of the two transitions (558 nm and 565 nm) it has been proposed by Slater and Lee[15] that they represent two species which operate at different levels of the phosphate potential. This conclusion is not supported by our studies (Figs 9A and 9B) which favour the theory that the spectrum of this species represents a twin hemochromogen as suggested by Sato *et al.*[5]. The stoichiometry of the peak area of the two transitions, *i.e.* 5:1 as calculated from Fig. 2A (lower curve), also supports this conclusion.

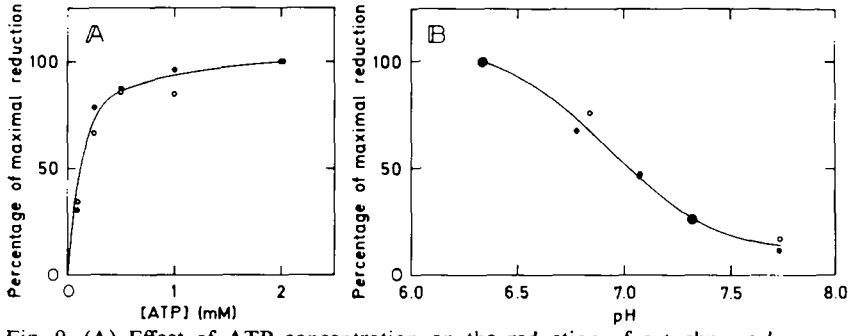


Fig. 9. (A) Effect of ATP concentration on the reduction of cytochrome $b_{565/558}$ as followed by the wavelength pairs 557 nm–575 nm (○) and 564 nm–575 nm (●). Except for the varying ATP concentrations the experiments were conducted as described in Fig. 1A. 0.59 mg of mitochondrial protein per ml. 100% reduction is defined as the steady-state redox level at 2 mM ATP in both experimental series. (B) Effect of pH on the reduction of cytochrome $b_{565/558}$ as followed by the wavelength pairs 557 nm–575 nm (○) and 564 nm–575 nm (●). Except for the varying pH values the experiments were conducted as described in Fig. 1A. 0.42 mg of mitochondrial protein per ml. 100% reduction is defined as the steady-state redox level at pH 6.34 in both experimental series.

In their model of electron transfer and energy conservation at Site 2, Chance *et al.*[3] have proposed that cytochrome b_{561} participates exclusively in electron transfer and is, therefore, termed b_K. This conclusion was based on their identification of the so-called cytochrome b_T (*i.e.* energy transducing cytochrome *b*) with cytochrome $b_{565/558}$[6] since only this species was found to be reduced by ATP in the presence of succinate. As shown in the present study, however, both cytochrome *b* species individually undergo redox reactions which are dependent on the phosphate potential which make it necessary to include cytochrome b_{561} also as an energy transducing cytochrome in this proposed model of energy transduction of the mito-

chondrial inner membrane. The tentative terms b_K and b_T in their original definitions[3] are, therefore, misleading and the different cytochrome *b* species should rather be termed according to the wavelength maxima of their α-bands only[30].

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