BBA 46031

HIGH-ENERGY FORMS OF CYTOCHROME *b*

II. THE EFFECT OF ATP AND ANTIMYCIN ON CYTOCHROME *b* IN INTACT MITOCHONDRIA

H. J. WEGDAM, J. A. BERDEN AND E. C. SLATER

Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)*

(Received July 30th, 1970)

SUMMARY

1. Both antimycin and ATP induce a red shift of the b band when added to succinate-reduced rat-heart mitochondria in the absence of oxygen. The intensity of the new band is greater with antimycin than with ATP, and the two effects are not additive, since ATP has only a slight effect in the presence of antimycin.

2. Both antimycin and ATP increase the degree of reduction of cytochrome *b*.

3. Energization of respiring mitochondria by adding oligomycin or of mitochondria suspended in a K^+-free medium by adding valinomycin is also associated with a red shift of the b band.

4. The curve describing the effect of different concentrations of antimycin on the red shift is sigmoidal in the presence of uncoupler and hyperbolic in the presence of ATP.

5. These results may be interpreted in terms of a mechanism for Site-II phosphorylation in which it is proposed that phosphorylation occurs in the reaction

$$b^{2+}\sim X\cdot b_i^{3+}\sim X + ADP + P_i \leftrightharpoons b^{3+}\cdot b_i^{2+} + 2X + ATP$$

6. The differences between intact mitochondria and phosphorylating sub-mitochondrial particles with respect to additivity of antimycin and ATP effects, and to the effect of ATP on the redox state of the cytochrome *b*, may be explained by assuming that $b_i^{3+}\sim X$ is unstable in sub-mitochondrial particles.

INTRODUCTION

According to the so-called chemical hypothesis of respiratory-chain phosphorylation[1,2], and in contradistinction to the chemiosmotic hypothesis[3], the primary energy-conserving reaction is the formation of a high-energy form of certain components of the respiratory chain. From early on, the possibility was entertained that cytochrome *b* might be one of these components[2,4]. The first direct evidence brought

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine.

* Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

forward in favour of this view was the observation by CHANCE AND SCHOENER[5] and CHANCE *et al.*[6] that, under certain circumstances, the addition of ATP to pigeon-heart mitochondria or sub-mitochondrial particles causes the appearance of an absorption band at a lower wavelength than normal at 77 °K (*viz.* at 555–556 nm).

Evidence of a different sort was provided by BONNER AND SLATER[7] and SLATER[8], who found that the shape of the antimycin-effect curve, describing the concentration dependence of the antimycin-induced "red shift", is sigmoidal in the presence of an uncoupler of oxidative phosphorylation and hyperbolic in the presence of ATP. The interpretation of this finding is that antimycin combines preferentially with an oligomeric form of cytochrome *b* present in high concentration in high-energy mitochondria and in low concentration in low-energy mitochondria.

An effect of the energetic condition of the mitochondria on the redox potential of cytochrome *b* was found by WILSON AND DUTTON[9]. Redox potentials (E_0' at pH 7.0) of 245 and 35 mV were measured in the presence of ATP, and of —55 and 35 mV in the presence of uncoupler.

Finally, SLATER *et al.*[10,11] have reported that in sub-mitochondrial particles isolated from beef heart, ATP brings about a shift to longer wavelengths (contrast CHANCE AND SCHOENER[5] and CHANCE *et al.*[6]) of the absorption maximum of ferrocytochrome *b*, as well as a slower rotenone-insensitive oxidation of the *b* in the presence of antimycin. The ATP-induced red shift is quantitatively the same both in the presence and absence of antimycin.

The present paper describes the effect of antimycin and of the energetic state on the absorption spectrum of cytochrome *b* in intact rat-heart and rat-liver mitochondria.

RESULTS

Effect of ATP and antimycin on cytochrome b spectrum in rat-heart mitochondria

In order to minimize effects of other cytochromes, cytochromes c_1, *c* and aa_3 were first reduced by adding ascorbate, tetramethyl-*p*-phenylene-diamine (TMPD) and cyanide (*cf.* CHANCE[12]). Reduction of cytochrome *b* by endogenous substrate was prevented by adding rotenone. Fig. 1A shows the spectra obtained, with 560 nm as reference wavelength, after the successive addition of succinate, ATP, antimycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and $Na_2S_2O_4$. With succinate alone, a maximum was obtained at 561.5 nm. Addition of ATP caused a slight shift to the red of the peak (to 562.5 nm) and a substantially increased reduction of cytochrome *b*, as indicated by the wavelength pair, 563–575 nm (see Table I). The subsequent addition of antimycin caused a further red shift (peak at 563.5 nm) and a slight further reduction. The subsequent addition of FCCP had little effect. There was a slight further increase in the degree of reduction on addition of $Na_2S_2O_4$, while the peak moved back towards the blue. Fig. 1B shows difference spectra describing the effects of ATP, ATP + antimycin, and of antimycin in the presence of ATP. Since the addition of antimycin after ATP caused an increased red shift with little increased reduction, the last curve describes more accurately the effect of adding antimycin on the spectrum of ferrocytochrome *b*. The peak is at about 565 nm.

Fig. 2 shows that when the order of addition of antimycin and ATP is reversed, ATP has little effect above that of antimycin.

Somewhat different results were obtained with glutamate and malate as substrate (see Table I). With both ATP and antimycin, cytochrome *b* was less reduced than with succinate. Furthermore, the red shift was less and, when ATP was added before antimycin, was not increased by the subsequent addition of antimycin. The difference between succinate and glutamate + malate may be related to the tighter coupling of phosphorylation at Site I than at Site II.

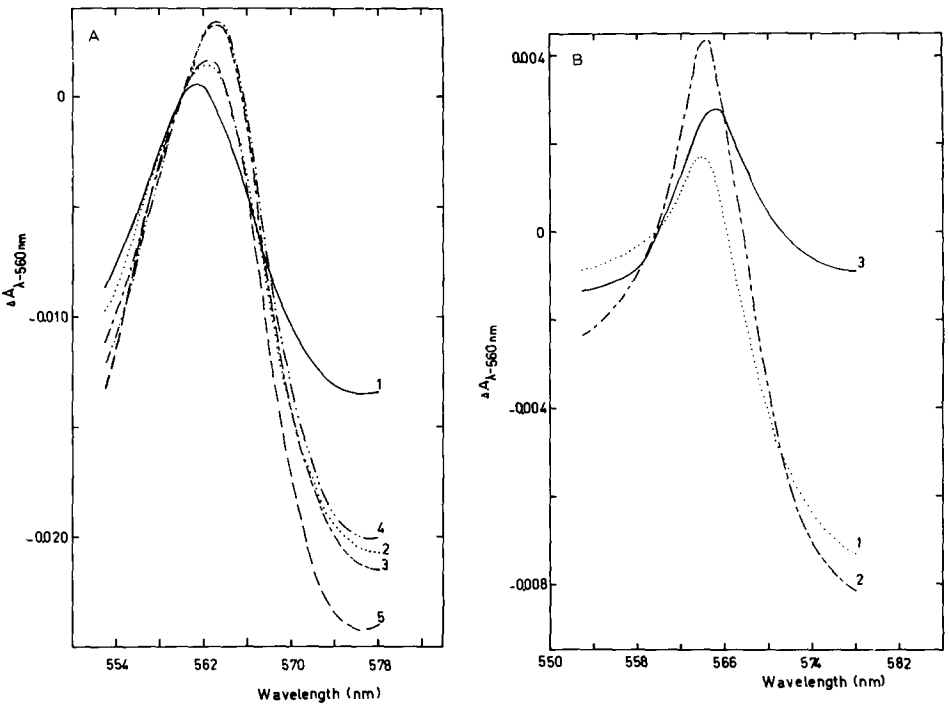

Fig. 1.A. Effect of successive additions of 20 mM succinate (Curve 1), 6 mM ATP (Curve 2), 1 nmole antimycin per mg protein (Curve 3), 8 mM FCCP (Curve 4) and $Na_2S_2O_4$ (Curve 5) on absorption spectrum of cytochrome *b* in rat-heart mitochondria (2 mg protein per ml) suspended in 30 mM glucose, 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl, and reduced with 8 mM ascorbate, 80 μM TMPD in the presence of 10 mM cyanide and 12 nmoles rotenone per mg protein. The spectra were measured in the dual-wavelength spectrophotometer, with 560 nm as reference wavelength, and are difference spectra drawn in relation to the situation before the addition of succinate. B. Difference spectra, calculated from A, describing: Curve 1, effect of ATP (difference between Curves 2 and 1 in A); Curve 2, effect of ATP + antimycin (difference between Curves 3 and 1 in A); Curve 3, effect of antimycin in the presence of ATP (difference between Curves 2 and 1 in B).

Effect of ATP on an antimycin-effect curve with rat-heart mitochondria

Fig. 3 shows that, as previously found with white-potato mitochondria[7,8], the antimycin-effect curve is linear (hyperbolic) in the presence of ATP and sigmoidal in the presence of uncoupler (FCCP). The maximal red shift brought about by ATP and antimycin was the same as that by antimycin alone (*cf.* Figs. 1 and 2).

Fig. 4 shows that, in the presence of uncoupler, the sigmoidicity is less marked in the case of the red shift than the effect on the degree of reduction of cytochrome *b*. (The wavelength pair used for studying the degree of reduction, 560.5–570 nm, is also

TABLE I

EFFECT OF ADDITION OF ATP, ANTIMYCIN, FCCP AND $Na_2S_2O_4$ ON REDUCTION OF CYTOCHROME *b* AND ON RED SHIFT IN RAT-HEART MITOCHONDRIA

Expt.	*Substrate*	*Successive additions*	*% Reduction of cytochrome b ($Na_2S_2O_4$ = 100)*	$\Delta A_{564.5-558.5\,nm} \times 10^3$
1	Succinate	Succinate	56	0
		ATP	85	(1.6)
		Antimycin	95	(5.1)
		FCCP	91	(5.2)
		$Na_2S_2O_4$	100	(−1.5)
2	Succinate	Succinate	52	0
		Antimycin	81	(5.3)
		ATP	87	(5.1)
		$Na_2S_2O_4$	100	(−1.0)
3	Glutamate–malate	Glutamate–malate	50	0
		ATP	72	(1.3)
		Antimycin	73	(2.6)
		FCCP	80	(3.5)
		$Na_2S_2O_4$	100	(3.6)

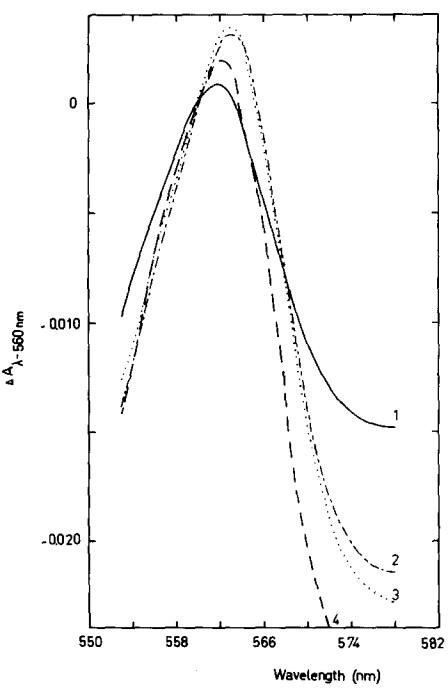

Fig. 2. Effect of successive additions of 20 mM succinate (Curve 1), 1 nmole antimycin per mg protein (Curve 2), 6 mM ATP (Curve 3) and $Na_2S_2O_4$ (Curve 4) on absorption spectrum of rat-heart mitochondria. Conditions otherwise as in Fig. 1.

affected by the red shift.) The same difference is also apparent with the Keilin and Hartree heart-muscle preparation[13].

Fig. 5 shows that, as is also the case with the Keilin and Hartree heart-muscle preparation[13], a hyperbolic curve is obtained with $Na_2S_2O_4$ as substrate, with both FCCP and ATP. The extent of the red shift is also less with $Na_2S_2O_4$ (see also Fig. 6).

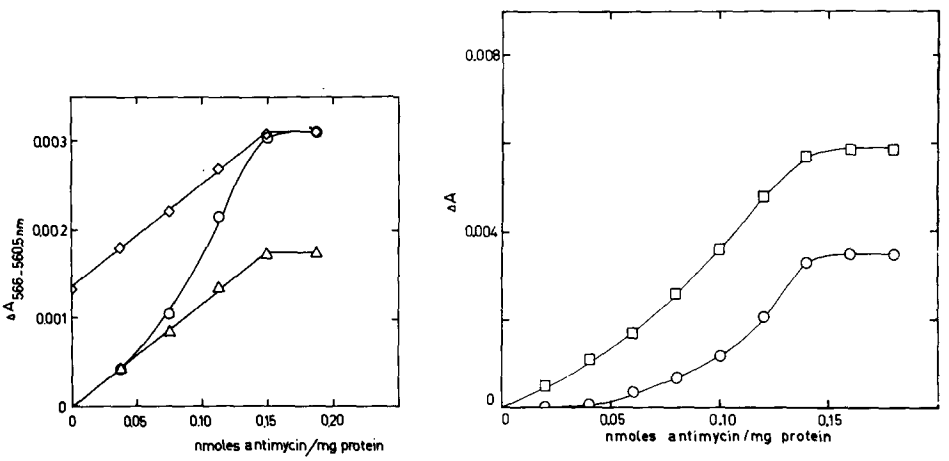

Fig. 3. Effect of concentration of antimycin on magnitude of red shift, measured as $\Delta A_{566-560.5\,nm}$, in rat-heart mitochondria (1.5 mg protein per ml) suspended in 45 mM sucrose, 50 mM Tris–HCl buffer (pH 7.5), 2 mM EDTA, 15 mM KCl, 5 mM $MgCl_2$ and 16 nmoles rotenone per mg protein. The reaction was started with 20 mM succinate. After the suspension became anaerobic, successive amounts of antimycin were added, and the increase in $\Delta A_{566-560.5\,nm}$ measured. ○—○, in presence of 12 μM FCCP; ◇—◇, 12 mM ATP added before antimycin; △—△, same as ◇—◇, but showing the effect of antimycin alone.

Fig. 4. Effect of concentration of antimycin on magnitude of red shift measured at 566–560.5 nm (□—□), and on degree of reduction of cytochrome *b* (○—○) in the presence of cyanide, measured at 570–560.5 nm. Rat-heart mitochondria (2.7 mg protein per ml) were suspended in 45 mM sucrose, 50 mM Tris–HCl buffer (pH 7.5), 2 mM EDTA, 15 mM KCl, 5 mM $MgCl_2$, 9 nmoles rotenone per mg protein, 12 mM ATP, 12 μM FCCP, 10 mM KCN, 8.0 mM ascorbate and 80 μM TMPD. Reaction started with 20 mM succinate, followed by successive amounts of antimycin.

Effect of energizing mitochondria by oligomycin or valinomycin on spectrum of cytochrome b

Mitochondria in the presence of substrate, oxygen, ADP and P_i are in a low-energy state and may be energized by adding oligomycin which inhibits the utilization for ATP synthesis of the primarily conserved energy. Fig. 7 shows that the addition of oligomycin to rat-heart mitochondria causes the reduction of a cytochrome *b* absorbing at a higher wavelength (563.5 nm) than that obtained by anaerobiosis (561.5 nm, see Fig. 1A).

Rat-liver mitochondria under anaerobic conditions are in an intermediate energy state, since they contain considerable amounts of endogenous ATP. When suspended in a K^+-free medium, they may be temporarily energized by addition of valinomycin which induces an outflow of endogenous K^+ from the mitochondria, in the direction of the K^+ gradient[14,15].

When ascorbate, TMPD and Na_2S are added to rat-liver mitochondria, the cytochrome *b* is partially reduced. This is revealed by a shoulder at 562 nm on the c band. The addition of succinate causes the further reduction of a cytochrome *b* absor-

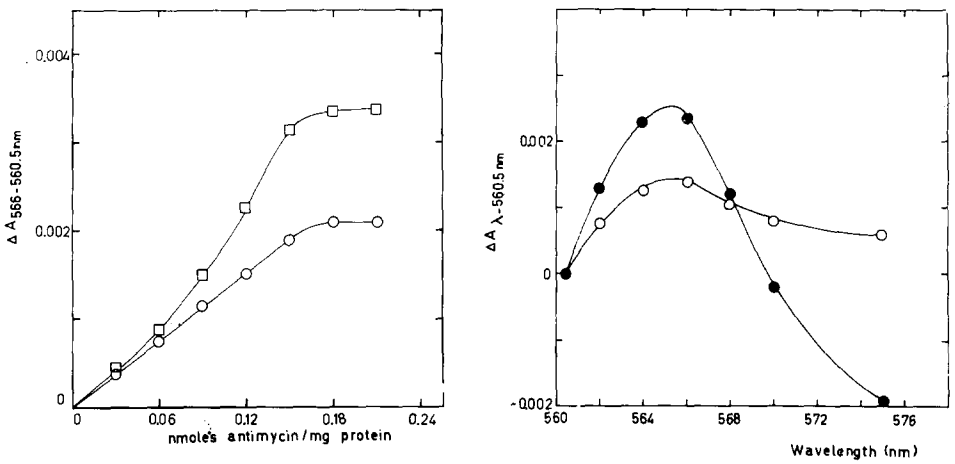

Fig. 5. Effect of concentration of antimycin on magnitude of red shift measured with succinate (□—□) and $Na_2S_2O_4$ (○—○) as reductant. Conditions as in Fig. 4. Rat-heart mitochondria, 2 mg protein per ml, 12 nmoles rotenone per mg protein.

Fig. 6. Effect of antimycin on absorption spectrum of cytochrome *b* in rat-heart mitochondria reduced by succinate or $Na_2S_2O_4$. Rat-heart mitochondria (1 mg protein per ml) suspended in 30 mM glucose, 30 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl, 0.5 mM ADP, 30 mM potassium phosphate (pH 7.5), 24 nmoles rotenone per mg protein and 13 units hexokinase. ●—●, reaction started by addition of 20 mM succinate. After the suspension became anaerobic, antimycin (4 nmoles/mg protein) was added, and the increased absorbance, with 560.5 nm as reference wavelength, recorded. Each point represents a different incubation. ○—○, same with $Na_2S_2O_4$ instead of succinate.

bing at 566 nm (see Fig. 8). This reduction of the long-wavelength cytochrome *b* appears to be dependent upon endogenous ATP, since it is reversed by addition of oligomycin (not shown in Fig. 8). The addition of valinomycin after succinate causes a further reduction of the long-wavelength cytochrome *b* (Fig. 8). This reduction is temporary. When the reduction induced by valinomycin has disappeared, it may be restored by ATP (□—□ in Fig. 8).

DISCUSSION

In the previous paper[11], it was shown that ATP can bring about a red shift in the maximum absorption of the α-band of ferrocytochrome *b* in phosphorylating sub-mitochondrial particles. In the present paper it is shown that energizing mitochondria by ATP, oligomycin or valinomycin (in a K^+-free medium) has the same effect. The effect of energizing the mitochondria on the various species of cytochrome *b* present in mitochondria will be discussed in terms of the mechanism for Site-II phosphorylation given in the previous paper[11].

When rat-heart mitochondria are reduced by succinate in the presence of ascorbate, TMPD and cyanide, they are in a low-energy state. One would expect, then, that the equilibrium of Reaction 5 would be to the right and that the reduction of cytochrome *b* might be described by the sum of Reactions 1–6, which is

$$b^{3+} \cdot b_i^{3+} + e \leftrightharpoons b^{2+} \cdot b_i^{3+}$$

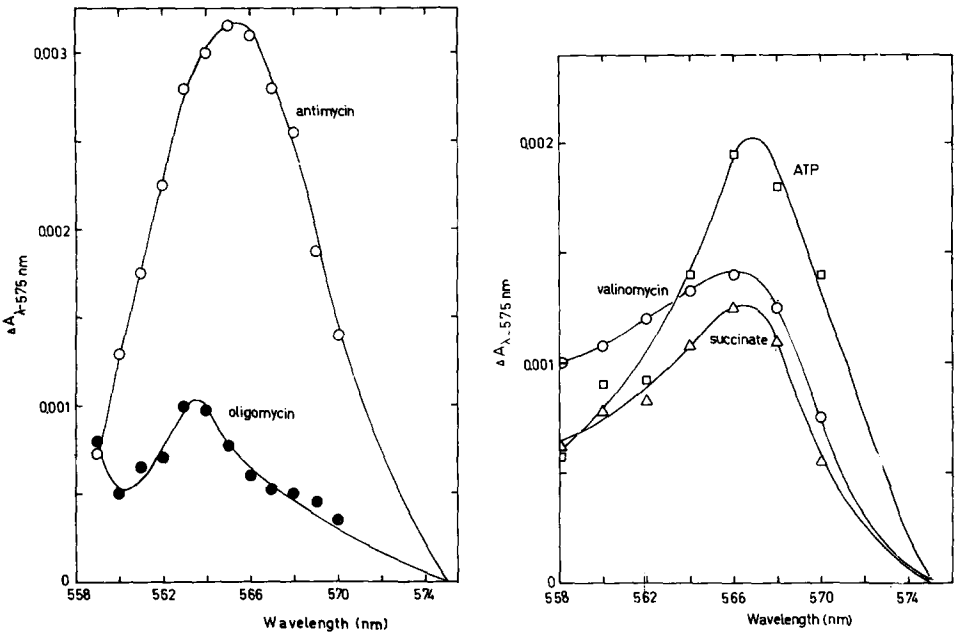

Fig. 7. Effect of oligomycin on the spectrum of cytochrome *b* in rat-heart mitochondria in State 3. Rat-heart mitochondria (1 mg protein per ml) were suspended in a medium containing 30 mM glucose, 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl, 4 mM phosphate, 1 mM ADP, 24 nmoles rotenone per mg protein, and 0.72 mM azide. The reaction was started with 20 mM succinate, and oligomycin (1.6 μg/mg protein) was added. ●—●, difference spectrum, with 575 nm as reference wavelength, oligomycin + succinate *minus* succinate. When anaerobiosis was reached, antimycin (3.8 nmoles/mg protein) was added. ○—○, difference spectrum, antimycin + oligomycin + succinate, anaerobic *minus* oligomycin + succinate, anaerobic. 0.72 mM azide was added in order to minimize the increase in reduction of cytochrome *c* and c_1 that occurs on the transition from State 3 to State 4 (*cf.* ref. 19). The points at each wavelenght were obtained in separate incubations.

Fig. 8. Effect of valinomycin on the redox state of cytochrome *b* in rat-liver mitochondria. Rat-liver mitochondria (1.6 mg protein per ml) were suspended in a medium containing 60 mM glucose, 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 2 mM EDTA, 15 nmoles rotenone per mg protein, 8 mM ascorbate, 80 μM TMPD and 2.4 mM Na_2S (brought to pH 7.5). Sodium salts were used and, where necessary, the pH was brought to 7.5 with aqueous NaOH. The reaction was started by the addition of 20 mM succinate; the spectrum △—△ shows the difference spectrum (reference wavelength, 575 nm), succinate *minus* before succinate. Valinomycin (0.5 μg/mg protein) was then added; the spectrum ○—○ shows the difference spectrum, immediately after valinomycin *minus* before valinomycin. The valinomycin-induced absorbance increase was only temporary. When the absorbance had returned to its original value, 12 mM ATP was added; the spectrum □—□ shows the difference spectrum, after ATP *minus* before ATP. The points at each wavelength were obtained in separate incubations.

In agreement with this prediction, cytochrome *b* is about 50 % reduced under these conditions (Table I).

The addition of ATP would be expected to drive Reaction 5, and therefore Reactions 6 and 4, to the left. The reactions observed would be, then,

$$b^{2+} \cdot b_i^{3+} \leftrightharpoons b^{3+} \cdot b_i^{2+}$$

$$b^{3+} \cdot b_i^{2+} + \text{ATP} + \text{X} + e \leftrightharpoons b^{2+} \sim \text{X} \cdot b_i^{2+} + \text{ADP} + \text{P}_i$$

One would expect an increased degree of reduction and a red shift due to the formation of $b^{2+}\sim X$, which is what is observed. Energization by oligomycin or valinomycin would have the same result.

The subsequent addition of antimycin could lead to the following reaction sequences.

$$b^{2+}\sim X\cdot b_i^{2+} + X \leftrightharpoons b^{2+}\sim X\cdot b_i^{3+}\sim X + e$$

$$b^{2+}\sim X\cdot b_i^{3+}\sim X + \text{antimycin} \longrightarrow b^{2+}\sim X\cdot (b_i^{3+}\sim X)\cdot\text{antimycin}$$

$$b^{2+}\sim X\cdot (b_i^{3+}\sim X)\cdot\text{antimycin} + e \leftrightharpoons b^{2+}\sim X\cdot (b_i^{2+}\sim X)\cdot\text{antimycin}$$

$$\text{Sum: } b^{2+}\sim X\cdot b_i^{2+} + X + \text{antimycin} \longrightarrow b^{2+}\sim X\cdot (b_i^{2+}\sim X)\cdot\text{antimycin}$$

Thus, according to this interpretation, spectrum No. 3 in Fig. 1B represents the red shift due to the conversion of b_i^{2+} into $b_i^{2+}\sim X$ (assuming that antimycin binding has no effect itself on the spectrum). These reaction sequences are consistent with the finding that antimycin, when added after ATP, increases the intensity of the red shift but not the degree of reduction.

In the absence of ATP, the addition of antimycin could also lead to the formation of $b^{2+}\sim X\cdot (b_i^{2+}\sim X)\cdot$antimycin by the same reaction sequence. Thus, in intact mitochondria, the same spectral shift is brought about by antimycin alone and by antimycin + ATP (Figs. 1 and 2). This is in contrast to sub-mitochondrial particles where the effects of ATP and antimycin are additive. We suggest that this difference is caused by the instability of $b_i^{3+}\sim X$ in sub-mitochondrial particles, leading to the following sequence of reactions on adding ATP to reduced particles.

$$b^{2+}\cdot b_i^{3+} \leftrightharpoons b^{3+}\cdot b_i^{2+}$$

$$b^{3+}\cdot b_i^{2+} + \text{ATP} + 2X \leftrightharpoons b^{2+}\sim X\cdot b_i^{3+}\sim X + \text{ADP} + P_i$$

$$b^{2+}\sim X\cdot b_i^{3+}\sim X \longrightarrow b^{2+}\sim X\cdot b_i^{3+} + X$$

$$b^{2+}\sim X\cdot b_i^{3+} \leftrightharpoons b^{3+}\cdot b_i^{3+} + X + e$$

In this way, both the ATP-induced red shift ($b^{2+}\cdot b_i^{3+} \longrightarrow b^{2+}\sim X\cdot b_i^{3+}$) and the subsequent oxidation of cytochrome b ($b^{2+}\sim X\cdot b_i^{3+} \longrightarrow b^{3+}\cdot b_i^{3+}$) are explained. b_i remains oxidized and plays no role of significance. Antimycin, by stabilizing $b_i\sim X$, brings this species into the reactions. In these particles, the ATP-induced reactions, which involve only b, and the antimycin-induced reactions, which involve only b_i, are additive.

In the previous paper[11], it is shown that ATP has no effect when sub-mitochondrial particles are reduced by $Na_2S_2O_4$. This is understandable since in the presence of such a strong reducing agent, both b and b_i will be completely reduced, and cannot be activated by ATP.

Thus, the mechanism proposed in the previous paper can explain the two differences between sub-mitochondrial particles and mitochondria observed, namely (i) the additivity of the antimycin- and ATP-induced red shift in sub-mitochondrial particles, and non-additivity in mitochondria; (ii) the increased oxidation of cytochrome b in sub-mitochondrial particles and increased reduction in intact mitochondria, brought about by addition of ATP.

METHODS

Rat-heart mitochondria were found more suitable than rat-liver mitochondria for three reasons. First, they have a higher concentration of cytochrome *b* which is useful considering the small absorbance differences measured in some types of experiments. Secondly, they show a higher rate of antimycin-insensitive respiration so that oxygen admitted with other reagents is rapidly consumed in experiments in which cyanide was not present (*cf.* ref. 11). Thirdly, rat-heart mitochondria, in the absence of oxidizable substrate or ATP, are less energized than rat-liver mitochondria.

Rat-heart mitochondria were prepared by the method of CLELAND AND SLATER[16], using a Potter–Elvehjem homogenizer.

Rat-liver mitochondria were prepared by the method of HOGEBOOM[17] as described by MYERS AND SLATER[18].

Protein was determined by the method of CLELAND AND SLATER[16].

An Aminco–Chance dual-wavelength spectrophotometer was used.

ACKNOWLEDGEMENTS

This work was supported by grants from the Life Insurance Medical Research Fund and from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

REFERENCES

1 E. C. SLATER, *Nature*, 172 (1953) 975.
2 B. CHANCE AND G. R. WILLIAMS, *Advan. Enzymol.*, 17 (1956) 65.
3 P. MITCHELL, *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Ltd., Bodmin, 1966.
4 E. C. SLATER AND J. P. COLPA-BOONSTRA, in J. E. FALK, R. LEMBERG AND R. K. MORTON, *Heamatin Enzymes*, Vol. 2, Pergamon Press, London, 1961, p. 575.
5 B. CHANCE AND B. SCHOENER, *J. Biol. Chem.*, 241 (1966) 4567.
6 B. CHANCE, C. P. LEE AND B. SCHOENER, *J. Biol. Chem.*, 241 (1966) 4574.
7 W. D. BONNER AND E. C. SLATER, *Biochim. Biophys. Acta*, 223 (1970) 349.
8 E. C. SLATER, *Koninkl. Ned. Akad. Wetenschap., Verslag Vergader. Afdel. Nat.*, 78 (1969) 123.
9 D. F. WILSON AND P. L. DUTTON, *Biochem. Biophys. Res. Commun.*, 39 (1970) 59.
10 E. C. SLATER, C. P. LEE, J. A. BERDEN AND H. J. WEGDAM, *Nature*, 226 (1970) 1248.
11 E. C. SLATER, C. P. LEE, J. A. BERDEN AND H. J. WEGDAM, *Biochim. Biophys. Acta*, 223 (1970) 354.
12 B. CHANCE, *J. Biol. Chem.*, 233 (1958) 1223.
13 J. BRYŁA, Z. KANIUGA AND E. C. SLATER, *Biochim. Biophys. Acta*, 189 (1969) 317.
14 R. S. COCKRELL, E. J. HARRIS AND B. C. PRESSMAN, *Nature*, 215 (1967) 1487.
15 E. ROSSI AND G. F. AZZONE, *European J. Biochem.*, 12 (1970) 319.
16 K. W. CLELAND AND E. C. SLATER, *Biochim. J.*, 53 (1953) 547.
17 G. H. HOGEBOOM, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 16.
18 D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558.
19 S. MURAOKA AND E. C. SLATER, *Biochim. Biophys. Acta*, 180 (1969) 227.