BBA 45809

THE REDOX STATES OF RESPIRATORY-CHAIN COMPONENTS IN RAT-LIVER MITOCHONDRIA

II. THE "CROSSOVER" ON THE TRANSITION FROM STATE 3 TO STATE 4

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(Received January 27th, 1969)

SUMMARY

1. When succinate or ascorbate *plus* TMPD is used as substrate, all electron carriers from NADH to cytochrome a_3 become more reduced on the transition from State 3 (ADP present) to State 4 (ADP consumed). It is concluded that the third site of respiratory control is located between cytochrome a_3 and oxygen.

2. β-Hydroxybutyrate and pyruvate give a crossover point between cytochromes *c* and *a*, and glutamate between cytochromes *b* and *c* or between cytochromes *c* and *a*. A crossover point between *a* and a_3 was also obtained with all NAD-linked substrates.

3. In the presence of increasing amounts of azide, hydroxylamine or cyanide, crossover points between cytochromes *c* and *a*, cytochromes *b* and *c*, and NADH and flavoprotein were found with β-hydroxybutyrate, and between cytochromes *c* and *a*, and cytochromes *b* and *c* with succinate. These results show that the first site of inhibition is located between substrate and flavoprotein and the second between succinate and cytochrome *c*.

4. It is concluded that a crossover point does not necessarily identify a site of inhibition in State 4.

INTRODUCTION

The respiratory rate of isolated mitochondria declines sharply when ADP, added to a phosphate-containing suspension medium, is consumed by being phosphorylated to ATP (ref. 1). These slowly respiring (State 4) mitochondria are characterized by a relatively high degree of reduction of respiratory-chain components near substrate[1–3]. On the addition of ADP, components near substrate become more oxidized, and those near oxygen more reduced[1–3]. The point between adjacent components where the component on the substrate side becomes more oxidized, and that

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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on the oxygen side more reduced, was named by CHANCE AND WILLIAMS[1] the crossover point.

The crossover point has been reported to be between cytochromes *c* and *a* in rat-liver mitochondria oxidizing succinate[4] or β-hydroxybutyrate[4,5], in guinea-pig-liver[4] and pigeon-heart mitochondria[6] oxidizing succinate, in rat-heart mitochondria oxidizing succinate[3] and in locust flight-muscle mitochondria oxidizing glycerol 1-phosphate[2]. A crossover point between cytochromes *b* and *c* was obtained with guinea-pig-liver mitochondria oxidizing glutamate[4], and with rat-heart mitochondria oxidizing glutamate *plus* malate[3]. CHANCE AND WILLIAMS[5] showed a shift of the crossover point towards substrate on the addition of azide or cyanide. In this way crossovers were found between cytochromes *b* and *c*, and between NADH and flavoprotein, with rat-liver mitochondria oxidizing β-hydroxybutyrate.

CHANCE AND WILLIAMS[1] proposed that the three crossover points detected under different conditions, *i.e.* between NADH and flavoprotein, cytochromes *b* and *c*, and cytochromes *c* and *a* represent sites at which the respiratory chain is inhibited in State 4, *i.e.* sites of interaction with ADP. On the other hand, SLATER[7] concluded that it is not possible to equate the crossover point with the inhibition site in a respiratory chain with multiple inhibition sites, since the net change of redox state of a particular carrier is determined by effects on carriers both before and after it in the respiratory chain. CHANCE *et al.*[8], however, stated that a computer solution of differential equations, derived upon the assumption that the law of mass action applies to the reactions of the components of the respiratory chain, supported the crossover theorem as proposed by CHANCE AND WILLIAMS[1].

In the previous paper[9], it is concluded that the redox state of an electron carrier in State 3, in which the respiratory rate is independent of ADP or P_i concentration, represents a kinetic steady state governed by the relative activities of those portions of the chain responsible for reduction and oxidation of the carrier (*cf.* ref. 10). In State 4, however, the respiratory chain is near thermodynamic equilibrium with ADP, P_i and ATP (*cf.* refs. 2, 3). According to this view, the transition from State 3 to State 4 is one from a kinetic steady state to near thermodynamic equilibrium. This throws a new light on the crossover phenomenon, and made a further examination of the crossover desirable.

RESULTS

Ascorbate plus TMPD as substrate

Fig. 1 shows that the addition of ADP to rat-liver mitochondria in the presence of ascorbate + TMPD causes an oxidation of both cytochrome *c*, measured at 550 mμ *minus* 540 mμ, and aa_3, measured at 445 mμ *minus* 455 mμ. In order to eliminate the possibility that redox changes of cytochrome *b* seriously interfere with measurements of cytochrome aa_3 with this wavelength pair, 0.06 μg antimycin per mg protein was added to block the interaction between cytochromes *b* and *c*, and 0.06 μg rotenone per mg protein to minimize the effect of endogenous substrates. Even under these conditions, where the redox state of cytochrome *b* does not change, cytochrome aa_3 becomes more oxidized on adding ADP. Since on the transition from State 3 to 4, the absorbance increases both at 445 mμ *minus* 455 mμ, where cytochromes *a* and a_3 contribute to the reduced *minus* oxidized spectrum in the ratio of 0.77, and at

605 mμ *minus* 590 mμ (see Fig. 2), where the relative contributions are 13 (A. O. MUIJSERS, personal communication), it is clear that both cytochromes *a* and a_3 become reduced on this transition. Fig. 2 shows that, in the absence of antimycin and rotenone, NAD, Q, cytochromes *b*, *c*, *a* and a_3 all become more reduced, whereas NADP is virtually completely reduced in both State 3 and State 4. In other words no crossover point, as defined by CHANCE AND WILLIAMS[1], is observed.

Succinate as substrate

Fig. 3 shows that also with succinate as substrate no crossover is observed. At all concentrations of succinate used (between 1 and 10 mM) cytochrome aa_3 became

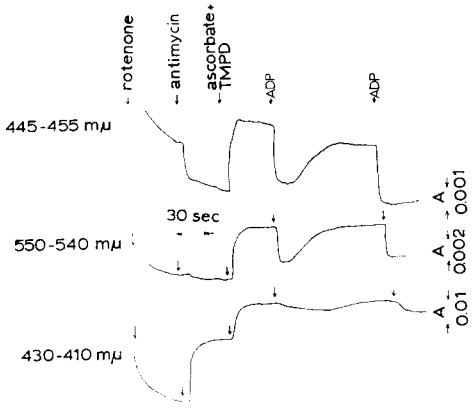


Fig. 1. Redox changes of cytochromes $a_3 + a$ (445–455 mμ), *c* (550–540 mμ) and *b* (430–410 mμ) by addition of ADP (100 μM) to mitochondria oxidizing 6 mM ascorbate *plus* 60 μM TMPD in the presence of 0.06 μg rotenone and 0.06 μg antimycin per mg protein. The reaction mixture contained 10 mM P_i and 1.9 mg/ml rat-liver mitochondria.

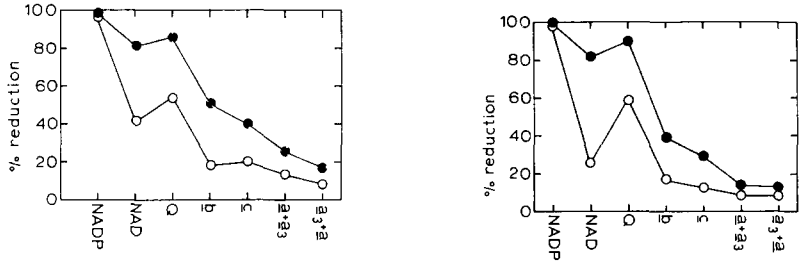


Fig. 2. Redox states of electron carriers in State-4 and State-3 mitochondria oxidizing ascorbate *plus* TMPD. In the measurements of the redox states of the cytochromes, the reaction mixture contained 0.1 mM ADP, 10 mM P_i, 6 mM ascorbate, 60 μM TMPD and 1.3 mg/ml rat-liver mitochondria. States 3 and 4 measured as in Fig. 4. The wavelength pair 605 mμ *minus* 590 mμ was used for cytochrome $a + a_3$. In the measurements of NAD, NADP and Q, the reaction mixture contained 0 (State 4) or 1 (State 3) mM ADP, 10 mM P_i, 6 mM ascorbate, 60 μM TMPD and 5.2 mg/ml mitochondria. ●—●, State 4; ○—○, State 3.

Fig. 3. Redox states of electron carriers in State-4 and State-3 mitochondria oxidizing succinate. In the measurement of redox states of the cytochromes, the reaction mixture contained 0.3 mM ADP, 10 mM P_i, 10 mM succinate and 1.7 mg/ml rat-liver mitochondria. State 3 and State 4 measured as in Fig. 4. The wavelength pair 605–590 mμ was used for cytochromes $a + a_3$. In the measurements of NAD, NADP and Q, the reaction medium contained 0 (State 4) or 1 (State 3) mM ADP, 10 mM P_i, 10 mM succinate and 6.6 mg/ml mitochondria. ●—●, State 4; ○—○, State 3.

more reduced on the transition from State 3 to State 4 (Fig. 4), but this was more easily observable with the lower concentrations since, under these conditions, cytochrome aa_3 is more oxidized in the kinetic steady state (State 3).

The respiratory carriers were less reduced in State 4 with NAD-linked substrates, and crossover points were observed. These were between cytochromes *c* and *a*, and *a* and a_3 with β-hydroxybutyrate (Fig. 5) and pyruvate (Fig. 6), and between cytochromes *b* and *c*, and cytochromes *a* and a_3 with glutamate (Fig. 7). In other experiments, a crossover between cytochromes *c* and *a* was also found with glutamate.

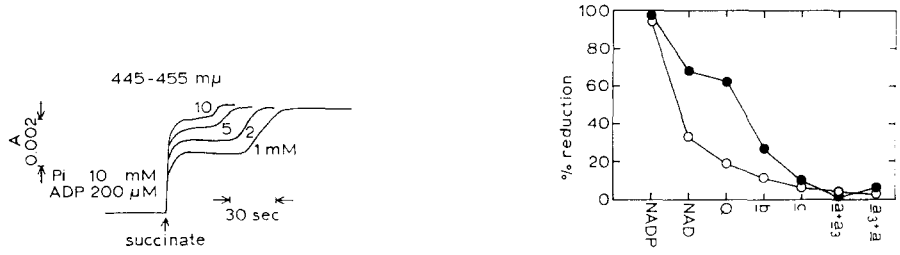


Fig. 4. Effect of succinate concentration on redox state of cytochromes $a_3 + a$ in State-3 and State-4 mitochondria. Succinate added in State 2 yielded first State 3 and then State 4. Concentrations of succinate (mM) are indicated. 0.1 μg/ml rotenone was present. 1.7 mg/ml rat-liver mitochondria.

Fig. 5. Redox states of electron carriers in State-4 and State-3 mitochondria oxidizing β-hydroxybutyrate. 10 mM β-hydroxybutyrate. 1.1 mg/ml mitochondria for the assay of cytochromes; 4.4 mg/ml mitochondria for the assay of NAD, NADP and Q. Other conditions as in Fig. 2. ●—●, State 4; ○—○, State 3.

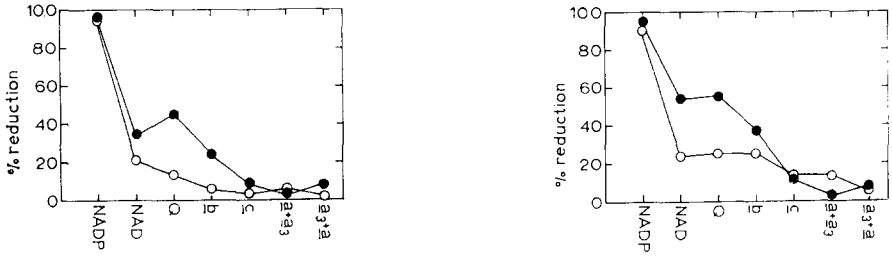


Fig. 6. Redox states of electron carriers in State-4 and State-3 mitochondria oxidizing pyruvate. 10 mM pyruvate. 1.8 mg/ml mitochondria for the assay of cytochromes; 7.2 mg/ml mitochondria for the assay of NAD, NADP and Q. Other conditions as in Fig. 2. ●—●, State 4; ○—○, State 3.

Fig. 7. Redox states of electron carriers in State-4 and State-3 mitochondria oxidizing glutamate. 10 mM glutamate. 1.3 mg/ml mitochondria for the assay of cytochromes; 5.2 mg/ml mitochondria for the assay of NAD, NADP and Q. Other conditions as in Fig. 2. ●—●, State 4; ○—○, State 3.

Effect of azide, hydroxylamine and cyanide

The effect of varying azide concentrations on the redox states with β-hydroxybutyrate as substrate is shown in Fig. 8. In agreement with the previous paper[9], azide has scarcely any effect in State 4, but in State 3 flavoprotein, cytochrome *b*, cytochrome *c* and cytochrome *a* become increasingly reduced with increasing concentrations of azide (*cf.* ref. 10). Thus, the crossover point lying between cytochromes *c* and *a* in the absence of azide shifts to between cytochromes *b* and *c* with 0.1 mM azide and

to between NADH and flavoprotein with 0.2 mM azide. The crossover point between cytochromes *a* and a_3 disappears with 0.2 mM azide. Cytochrome a_3 is more highly oxidized than cytochrome *a* in State 3 in the presence of azide[11–13].

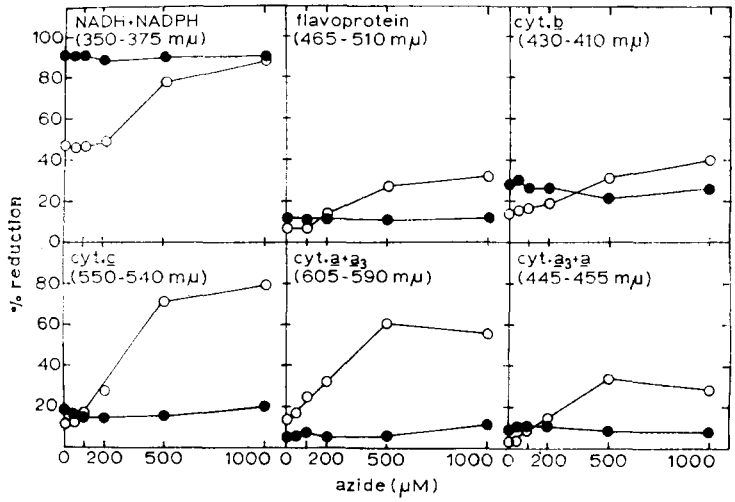


Fig. 8. Effect of azide concentration on redox states of electron carriers in State-4 and State-3 mitochondria oxidizing β-hydroxybutyrate. Reaction mixture contained 0.2 mM ADP, 10 mM P_i, 10 mM β-hydroxybutyrate and 1.2 mg/ml rat-liver mitochondria. No correction was made for the effect of azide on the absorption spectrum of ferrocytochrome *a* (refs. 11 and 12). ●—●, State 4; ○—○, State 3.

Fig. 9 shows the effect of varying azide concentrations with succinate as substrate. With 50 μM azide, the pattern changes to that observed with NAD-linked substrates in the absence of azide, namely crossover points between cytochromes *c* and *a*, and *a* and a_3. With 0.2 mM azide, a single crossover between cytochromes *b*

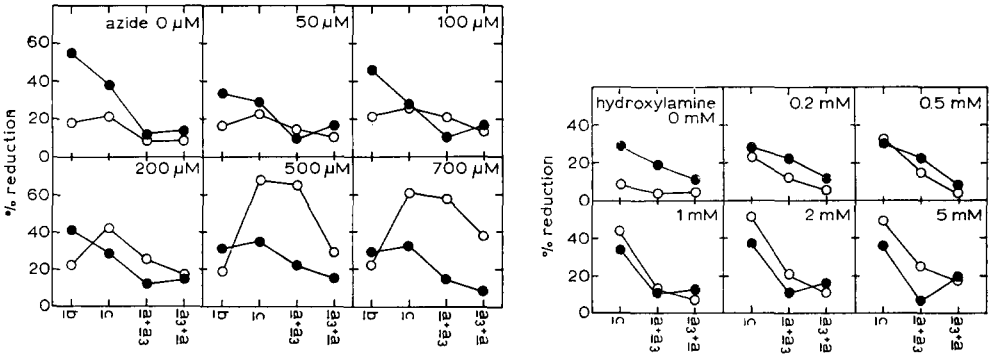


Fig. 9. Effect of azide concentration on redox states of electron carriers in State-4 and State-3 mitochondria oxidizing succinate. 10 mM succinate. 1.3 mg/ml rat-liver mitochondria. Other conditions as in Fig. 8. ●—●, State 4; ○—○, State 3.

Fig. 10. Effect of hydroxylamine concentration on redox states of electron carriers in State-4 and State-3 mitochondria oxidizing succinate. 1.3 mg/ml rat-liver mitochondria. Reaction mixture contained 0.2 mM ADP, 10 mM P_i, 10 mM succinate and 1.3 mg/ml rat-liver mitochondria. ●—●, State 4; ○—○, State 3.

and *c* appears. Cyanide behaved similarly to azide. Hydroxylamine, recently studied by WIKSTRÖM[14], also behaved rather similarly to azide, crossover points appearing between cytochromes *c* and *a*, and *a* and a_3 (Fig. 10).

DISCUSSION

Five different crossover phenomena have been identified in this investigation, namely:

(1) no crossover was observed with succinate or TMPD as substrate, in the absence of respiratory inhibitor;

(2) a crossover was observed between cytochromes *a* and a_3 with NAD-linked substrates in the absence of inhibitor, and with succinate in the presence of 50 μM azide or 1 mM hydroxylamine;

(3) a crossover was observed between cytochromes *c* and *a* with NAD-linked substrates in the absence of inhibitor, and with succinate in the presence of 50 μM azide or 0.5 mM hydroxylamine;

(4) a crossover was observed between cytochromes *b* and *c* with glutamate in the absence of inhibitor, and with succinate and β-hydroxybutyrate in the presence of 0.2 and 0.1 mM azide, respectively;

(5) a crossover was observed between NADH and flavoprotein with β-hydroxybutyrate in the presence of 0.2 mM azide.

Three of the five phenomena have been identified earlier[1-6,11,13]. The observation that all components of the respiratory chain, including cytochromes *a* and a_3, become reduced on the transition from State 3 to State 4 of mitochondria oxidizing succinate or TMPD is new. A crossover between cytochromes *a* and a_3, in the absence of inhibitor, has also not been observed earlier.

For a discussion of the crossover phenomenon the following respiratory chain will be assumed.

$$SH_2 \rightarrow NAD^+ \rightarrow fp \rightarrow Q \rightarrow b \rightarrow c_1 \rightarrow c \rightarrow a \rightarrow a_3 \rightarrow O_2$$

(succinate; TMPD; ascorbate)

SH_2 represents NAD-linked substrate, fp the two flavoproteins[15] involved in the oxidation of NADH, *b*, c_1, *c*, *a* and a_3 the corresponding cytochromes.

CHANCE AND WILLIAMS[1] introduced the important concept that, in State 4, the respiratory chain is inhibited by the lack of ADP required for the phosphorylating reactions of the respiratory chain. When there is only one site of interaction with ADP, as with TMPD as substrate in the presence of antimycin and rotenone, the interpretation of the crossover phenomenon is straightforward. The observation that all components of the respiratory chain become more reduced on the transition from State 3 to State 4 localizes the reaction between ferrocytochrome a_3 and oxygen as the site of inhibition.

Where there is more than one site of interaction with ADP, the interpretation is more difficult. The observation that all components of the respiratory chain become more reduced on the transition from State 3 to State 4, also with succinate as substrate,

leads to the same conclusion as with TMPD. Similarly, the finding that, in mitochondria oxidizing β-hydroxybutyrate in the presence of 0.2 mM azide, flavoprotein becomes more oxidized on the transition from State 3 to State 4 shows that the reaction between substrate and flavoprotein is a site of inhibition. However, in our view (see ref. 7) crossovers within the span between flavoprotein and cytochrome a_3, observed under various conditions with NAD-linked substrates, cannot be identified with inhibitory sites. For example, the redox state of cytochrome *c* depends upon the activities of the segments of the chain responsible for the reduction of ferricytochrome *c* by substrate, and for the oxidation of ferrocytochrome *c* by oxygen. We have just concluded that both segments are inhibited on the transition from State 3 to State 4. Whether cytochrome *c* will become more oxidized or reduced will depend upon which of the two influences it is subjected to dominates. In rat-liver mitochondria oxidizing β-hydroxybutyrate in the absence of inhibitor, it appears that inhibition of the oxidation of cytochrome *c* has the more important influence since the cytochrome becomes more reduced on the transition from State 3 to State 4 (Fig. 5). When, however, the oxidation step is slowed with azide, the cytochrome *c* becomes more susceptible to inhibition of the reduction of cytochrome *c*, and the cytochrome becomes more oxidized on the transition (Fig. 8). The experiment illustrated in Fig. 11 is particularly instructive in this respect. With TMPD as substrate, the transition from State 3 to State 4 leads to reduction of cytochrome *c* both in the presence and absence of azide, because only the span cytochrome *c* to oxygen becomes inhibited as the ADP is exhausted. With succinate as substrate, under otherwise identical conditions, cytochrome *c* becomes more oxidized on the transition from State 3 to State 4 in the presence of 0.2 mM azide, because the redox state of cytochrome *c* becomes more susceptible to the inhibitory effect on its reduction. This is a clear demonstration that with succinate as substrate an interaction with ADP takes place that is not observed with TMPD. However, the crossover between cytochromes *a* and a_3 observed under these conditions (see Fig. 9) does not locate the inhibition site between cytochromes *a* and a_3. In our view, the only conclusion to be drawn is that inhibition occurs between substrate and cytochrome a_3. Similarly, the only conclusion to be drawn from the

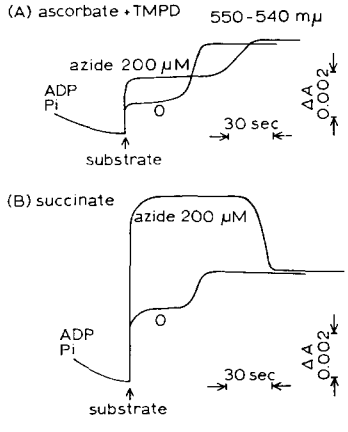


Fig. 11. Dependence of crossover point on substrate. Measurements at 550 mμ *minus* 540 mμ. 1.6 mg/ml rat-liver mitochondria. Additions of 10 mM succinate, or 5 mM ascorbate *plus* 0.15 mM TMPD, and of ADP (0.2 mM with succinate, 0.1 mM with TMPD) as shown.

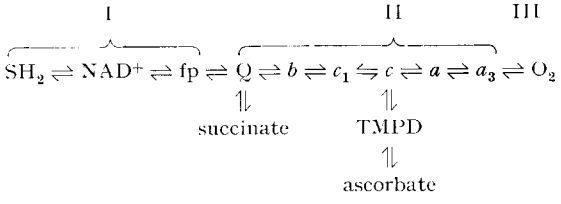
second crossover between cytochromes *c* and *a* is that there is an inhibition site between cytochrome *c* and oxygen.

Thus, the fact that five crossover sites have been identified under different conditions does not necessarily mean that there are five sites of inhibition in State-4 mitochondria.

A crossover point on the substrate side of cytochrome *c* was observed with succinate in the presence of 0.2 mM azide. This identifies a crossover between succinate and cyrochrome *c*. No crossover below cytochrome *c* was ever observed with TMPD as substrate, or below ubiquinone with succinate.

Thus, the observed crossover phenomena are consistent with the idea that the three sites of respiratory-chain phosphorylation lie between (i) NAD-linked substrate and flavoprotein, (ii) the point at which reducing equivalents from succinate enter the respiratory chain and cytochrome *c*, and (iii) cytochrome a_3 and oxygen. This is the first direct evidence identifying the latter region of the chain as a phosphorylating site, although this was already very likely on theoretical grounds[15]. Moreover, RAMIREZ[16] reported a crossover beyond cytochrome a_3 on initiation of muscle activity in intact toad, frog or lobster heart muscle.

The nature of the inhibitory reaction in State 4 has not been specified in the above discussion. In the previous paper[9], it was concluded that in State 4 the respiratory chain is near thermodynamic equilibrium with ADP, ATP and P_i. The respiratory chain in State 4 should, then, be written



The Roman numerals indicate the regions of the interaction with one molecule of ADP and P_i per two electrons. When ADP is added, the position of equilibrium of those reactions in which there is interaction with ADP is driven so far to the right that thermodynamic equilibrium is not reached, and the redox state of each carrier is governed by a kinetic steady state.

EXPERIMENTAL

The method of preparing the mitochondria and the composition of the reaction mixture are given in the previous paper[9].

The redox state of nicotinamide nucleotides (NAD + NADP), flavoprotein and the cytochromes was measured as described by CHANCE AND WILLIAMS[4], using an Aminco-Chance dual-wavelength spectrophotometer. The following wavelength pairs were used: nicotinamide nucleotides, 350 mμ *minus* 375 mμ; flavoprotein, 465 mμ *minus* 510 mμ; cytochrome *b*, 430 mμ *minus* 410 mμ; cytochrome *c*, 550 mμ *minus* 540 mμ; cytochrome $a + a_3$, 605 mμ *minus* 590 mμ, or 605 mμ *minus* 630 mμ; cytochrome $a_3 + a$, 445 mμ *minus* 455 mμ. The absorbance at these wavelengths was

measured during the sequence States $2 \rightarrow 3 \rightarrow 4 \rightarrow 5$, using the terminology of CHANCE AND WILLIAMS[1]. State 2 was induced by adding to the mitochondria, suspended in the reaction mixture, 10 mM P_i and an appropriate amount of ADP (0.2–0.3 mM for succinate and NAD-linked substrates, 0.1 mM for ascorbate and TMPD). State 3 was then induced by adding substrate. This was followed by State 4 when the ADP was consumed. State 5 (anaerobic) was induced by adding $Na_2S_2O_4$, except when nicotinamide nucleotides were measured. In this case, the suspension was allowed to go anaerobic by consumption of substrate. In calculating the percentage reduction, it was assumed that the carriers in State 2 were completely oxidized, and in State 5 completely reduced. When a respiratory inhibitor was present in the reaction mixture, the State-2 level was measured by adding 0.1–0.2 mM dicoumarol to the mitochondria in State 4.

The measurements at 465 mμ *minus* 510 mμ probably refer largely to the flavin moiety of NADH dehydrogenase, but the extent to which non-haem iron (*cf.* ref. 17) and other flavoproteins contribute is uncertain. The $\Delta\varepsilon_{mM}$ of cytochrome c_1 at 550 mμ *minus* 540 mμ in the difference spectrum reduced *minus* oxidized is 41% of that of cytochrome *c* (T. A. BERDEN, personal communication). Since mitochondria contain less cytochrome c_1 than cytochrome *c*, the absorbance changes with this wavelength pair can largely be ascribed to cytochrome *c*. The relative contributions of cytochromes *a* and a_3 to the measurements with the wavelength pairs 605 mμ *minus* 590 mμ, and 445 mμ *minus* 455 mμ have already been discussed. With the wavelength pair 605 *minus* 630 mμ, cytochrome *a* contributes about 3 times as much as cytochrome a_3 (A. O. MUIJSERS, personal communication).

The measurement of cytochrome *a* was especially difficult. CHANCE AND WILLIAMS have used both 590 mμ (ref. 1) and 630 mμ (ref. 4) as the reference wavelength. In Fig. 12, measurements at these wavelengths are compared, using a low concentration (1 mM) of succinate as substrate. Qualitatively, the same results were obtained with both wavelength pairs, *viz.* an increase of absorbance on transition from State 3 to State 4 and a decrease on the addition of ADP. However, there are quantitative differences which were found, moreover, to depend upon the age of the preparation. With aged preparations, the addition of ADP sometimes caused an increased

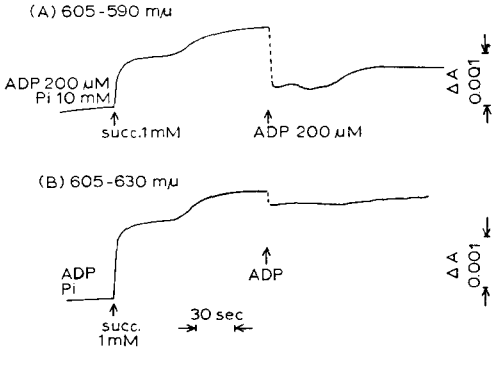


Fig. 12. Redox changes of cytochromes $a + a_3$ determined at 605 mμ *minus* 590 mμ or at 605 mμ *minus* 630 mμ on transition from State 3 to State 4 of rat-liver mitochondria oxidizing succinate (succ). Reaction medium contained 10 mM P_i and 0.1 μg/ml rotenone. 1.4 mg/ml rat-liver mitochondria.

absorbance at 605 *minus* 630 mμ. Since the changes of light absorption at this wavelength are very small, it is probable that light-scattering changes[18] interfere, even with measurements with the dual-wavelength spectrophotometer. Contraction of the mitochondria would cause a decreased value of $A_{605\,m\mu}$ *minus* $A_{590\,m\mu}$, thereby reinforcing the absorbance change on the addition of ADP, and an increased value of $A_{605\,m\mu}$ *minus* $A_{630\,m\mu}$, thereby nullifying the absorbance change. The effect of these light-scattering changes is very largely eliminated by measuring the average of the effects at the two wavelength pairs. In any case, it can safely be concluded from Fig. 12 that cytochrome *a* becomes more reduced on the transition from State 3 to State 4.

The degree of reduction of NAD and NADP was measured by the methods of KLINGENBERG[19,20], as described by VAN DAM[21]. The redox state of ubiquinone was measured by the extraction procedure of KRÖGER AND KLINGENBERG[22].

ACKNOWLEDGEMENTS

This work was supported in part by grants from the U.S. Public Health Service (Grant No. AM 08690) and the Life Insurance Medical Research Fund. We are grateful to Dr. R. D. VELDSEMA-CURRIE for her collaboration in the measurement of NAD, NADP and ubiquinone, and to Dr. K. VAN DAM for helpful discussions.

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