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Electron transport system in swine lung worm, *Metastrongylus elongatus*

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Introduction

Although electron transports and oxidative phosphorylations have been studied extensively in certain mammalian tissues, yeast and bacteria, relatively little is known about these processes in invertebrate metazoa. In heminthes, informations available have been confined to *Ascaris* worms which live under low oxygen tension (Kikuchi *et al.*, 1959; Kikuchi & Ban, 1961; Kmetec & Bueding, 1961; Chance & Parsons, 1963; Costello *et al.*, 1963; Chin & Bueding, 1954).

In previous papers (Ozawa *et al.*, 1964; 1965; 1966) the authors have shown that swine lung worms, which live under higher oxygen tension compared to the *Ascaris* worm, have aerobic respiration and that its mitochondria carry out oxidative phosphorylation similar to mammalian systems.

The present investigation was concerned with the further elucidation of components of electron transport system in lung worm mitochondria by spectrophotometric examinations. The results have demonstrated the existence of an electron transport system with cytochromes and ubiquinone in the lung worm mitochondria.

Materials and Methods

Preparation of mitochondria: The method of preparation of mitochondria from lung worms followed principally the previously described procedure with an exception that no bacterial proteinase was used (Ozawa & Sato, 1965). The isolation medium contained 0.3 M saccharose, 1 mM EDTA and a small amount of tris (hydroxymethyl) aminomethane to adjust the pH to around 7.2. Mitochondria obtained by 10,000 g 10 minute centrifugation were finally suspended in an appropriate volume of either 0.1 M phosphate buffer (pH 7.4) or 0.2 M Tris-HCl buffer (pH 7.4) and served as the stock suspension. When CO binding pigments were assayed, mitochondria were treated with 0.5% $NaNO_2$ in 0.1 M phosphate buffer solution for 5-10 minutes in the process of mitochondrial preparation in order to get rid of interference by hemoglobin (Chance, 1957).

Difference spectrum: Measurements of turbid suspension were made at room temperature in a Shimazu type MPS-50 multipurpose spectrophotometer. Equal concentration of aerated and substrate-free mitochondrial suspension was placed in two cuvettes of 1 cm optical path, and a base line was drawn. Then a small amount (ca. 5 μl) of saturated succinate (ca. 2 M) was added to one cuvette. After oxygen was depleted, the trace corresponding to reduced minus oxidized spectrum was drawn. When CO spectrum was to be examined, substrate was added to both cuvettes in advance so that their content became reduced. After a base line was drawn, the content of one cuvette was bubbled for 1 minute with CO and thereafter the trace corresponding to reduced+CO minus reduced spectrum was drawn.

Ferrocytochrome c oxidase activity: Activity was followed spectrophotometrically by measuring the decrease in absorbance at 550 mμ (19-21°C) according to Smith &

Conrad (1956). Ferricytochrome c in 0.1 % NaCl was reduced by addition of dithionite followed by gassing with air (87–94 % reduction). The complete assay system contained 2.9 ml of 0.08 M phosphate buffer (pH 7.0), 0.1 ml of 15–19.7 μM ferrocytochrome c solution. Reaction was begun by the addition of an appropriate amount of mitochondria (0.16–0.37 mg protein/ml of reaction medium).

NADH- and succinate cytochrome c reductase activity: Activities were followd by measuring the increase in absorbance at 550 mμ in the identicl system as ferrocytochrome c oxidase measurement in the presence of excess NADH or succinate after the oxidase was inhibited by the addition of 0.02 ml of 10^{-1} M KCN (neutralized).

Steady state measurement of ubiquinone: Ubiquinone determination in mitochondrial suspension followed principally the method described by Pumphrey & Redfearn (1960). Because of the low ubiquinone level in lung worm mitochondria (Sato & Ozawa, 1969), the volume of the suspension was doubled. Mitochondrial suspension, which was aged in ice box (0–4°C) for 1–2 days and free of endogenous substrate, was aerated with O_2 for 2 minutes so that the endogenous ubiquinone became oxidized form. Portions (2 ml) of the mitochondrial suspension containing 36–49.4 mg protein/ml in 0.2 M Tris-HCl buffer were incubated for 5 minutes (37°C) in the presence of succinate (50 μ-moles) alone or succinate and an inhibitor. Then the mixture was shaken for 1 minute to bring the respiratory carriers to their steady state level. The mixture was rapidly denatured by addition of 8 ml methanol at −20°C containing 0.1 % pyrogallol. Quinone was extracted twice with 10 ml light petroleum and after treatment of the combined light petroleum extracts with 95 % methanol, light petroleum layer was evaporated under reduced pressure. Ubiquinone was estimated by the difference in absorption between oxidized and borohydride reduced form in ethanol at 275 mμ.

Protein determination: Protein content of mitochondrial suspension was determined by the biuret method as described in the previous paper (Ozawa & Sato, 1965).

Chemicals: Cytochrome c (horse heart) from Daiichi Pure Chemical Co., NADH from Sigma Chemical Co., and antimycin A from Kyowa Hakko Co. were used. All other chemicals used were reagent grade.

Results

Difference spectrum of lung worm mitochondria.

When the difference in absorption was measured between the oxidized lung worm mitochondria and mitochondria that had been reduced by the addition of respiratory substrate, a cytochrome system similar to that of mammalian mitochondria was revealed (Fig. 1). When the measure sample was reduced with succinate as substrate (Fig. 1 A), the α region of the spectrum revealed a reduced cytochrome a absorption with a maximum at around 600 mμ, and reduced cytochrome b appeared as an absorption band with a maximum at 560 mμ; while reduced cytochrome c showed an absorption band with

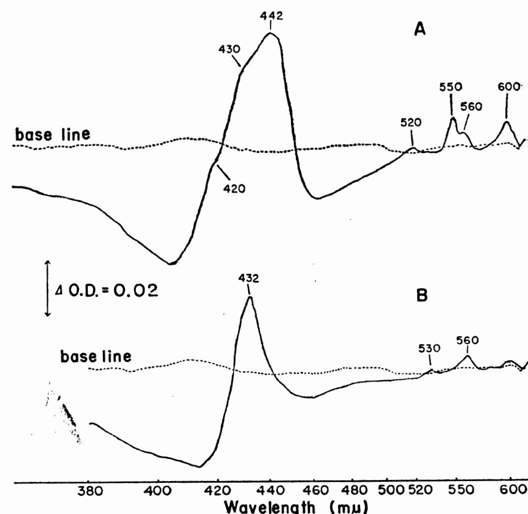

Fig. 1 Difference spectra (reduced minus oxidized) of lung worm mitochondria.
A: Succinate reduced minus oxidized difference spectrum. B: Difference spectrum in the presence of antimycin A (2 μg/ml). Each cuvette contained 3 ml of mitochondrial suspension equivalent to about 3 g of lung worms.

a maximum at 550 mμ. The β band at 520 mμ was typical of reduced cytochrome c. The Soret region of the spectrum showed an absorption band that was a composite consisting of reduced cytochrome a ($+a_3$) as a peak at 442 mμ and reduced cytochrome b and c as shoulders at 430 mμ and 420 mμ respectively.

Flavin, as flavinadeninedinucleotide, has an absorption minimum at 445 mμ in difference spectrum, so an absorption minimum in the region of 460 mμ may be attributable to flavoprotein.

Prior addition of antimycin A to the measure sample resulted in the reduction of cytochrome b and the oxidation of cytochrome c and a, as was demonstrated in the spectrum showing maxima at 560 mμ, 530 mμ and 432 mμ for the α, β and Soret maxima of cytochrome b (Fig. 1 B).

In the succinate reduced minus oxidized difference spectrum shown above, an absorption peak of a-type cytochrome shifted somewhat to shorter wavelengths than that of a typical one (605 mμ). Then, in reduced+CO minus reduced difference spectrum the property of a-type cytochrome was further investigated (Fig. 2).

While a trough at 443 mμ due to the Soret absorption band of reduced cytochrome a_3 appeared, a maximum of CO binding pigment appeared at 420 mμ rather than at 430 mμ, a typical absorption maximum of CO bound cytochrome a_3. When the mitochondrial suspension was pretreated with $NaNO_2$ as described in Materials and Methods, the result was the same. Moreover, when cytochrome a and c were in oxidized form upon a prior addition of antimycin A as in the case of Fig. 1 B, bubbling of CO into the measure sample resulted in a spectrum showing a small maximum at around 420 mμ and no trough at 445 mμ (Fig. 2 B). Hence, it is likely that the maximum at 420 mμ in the CO difference spectrum is attributable to the contamination effect of a hemoglobin like

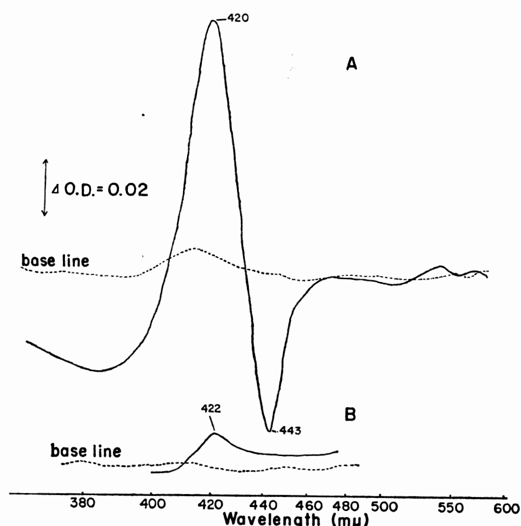

Fig. 2 Difference spectra showing the effect of carbon monoxide on lung worm mitochondria.
A: Succinate reduced+CO minus succinate reduced difference spectrum. B: CO difference spectrum in the presence of antimycin A (2 μg/ml). After both test and reference samples were reduced by addition of succinate, CO was bubbled through the test cuvette.

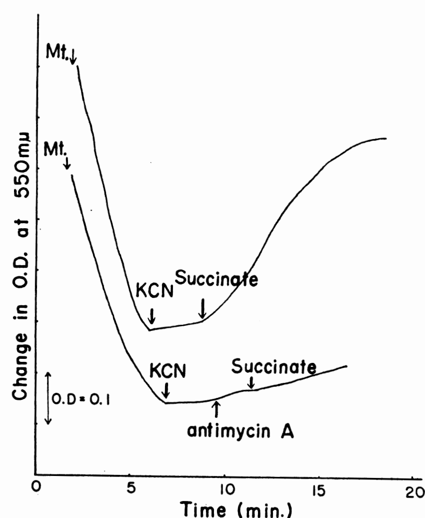

Fig. 3 Spectrophotometric traces showing cytochrome c oxidase and succinate cytochrome c reductase activity of lung worm mitochondria.
At arrows mitochondrial suspension (1.1 mg protein), KCN (6.6×10^{-4} M), succinate (4 mM) and antimycin A (2.7 μg/ml) were added respectively.

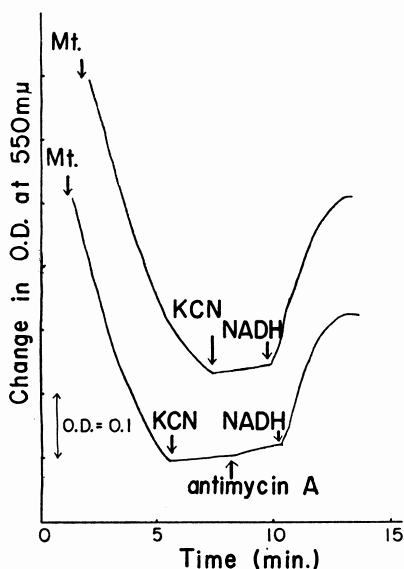

Fig. 4 Spectrophotometric traces showing cytochrome c oxidase and NADH cytochrome c reductase activity of lung worm mitochondria. Reaction system was the same as in Fig. 3 except for NADH (0.39 mM) was used as substrate in the place of succinate.

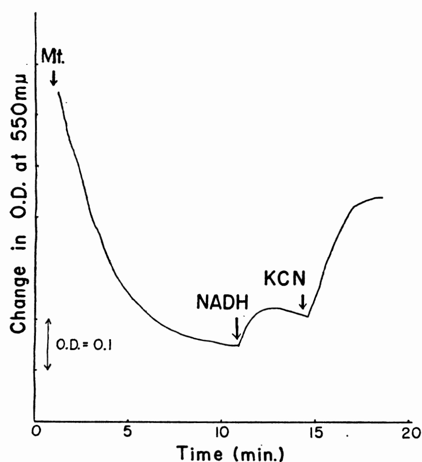

Fig. 5 Effect of KCN on the reduction of cytochrome c by exogenously added NADH.

substance in spite of the $NaNO_2$ treatment (Chance, 1957).

Enzyme activity.

Sequence of electron transport components in the lung worm mitochondria was further studied by following the enzymatic activities of mitochondria on exogenously added cytochrome c.

Fig. 3 illustrated the change in optical desity following the addition of mitochondria to reduced cytochrome c. There was a decrease in optical density as the reduced cytochrome c was oxidized. Addition of cyanide abolished further oxidation.

Table 1 Cytochrome c oxidase activity of lung worm mitochondria

case	Activity cytochrome c oxidized μmole/hr. mg protein
6	1.47 (1.03∼2.31)

The complete assay system contained 2.9 ml of 0.08 M phosphate buffer (pH 7.0), 0.1 ml of 15∼19.7 μM ferrocytochrome c (87∼94% reduction) and an appropriate amount of mitochondria (0.48∼1.11 mg protein). The reaction was carried out at 19∼21°C.

Table 1 shows the specific activity of cytochrome c oxidase of lung worm mitochondria.

Using the identical system as cytochrome c oxidase assay, NADH-cytochrome c reductase and succinate cytochrome c reductase activity were followed in the presence of cyanide. As shown in Fig. 3 and Fig. 4, whereas the latter was inhibited by antimycin A, the former was antimycin A insensitive. As shown in Fig. 5 this antimycin A insensitive reduction of cytochrome c by NADH became more prominent in the presence of cyanide than in the absence of it, which may imply that cytochrome c reduced by exogenously added NADH is reoxidized through a cyanide sensitive oxidase.

Steady state oxidation-reduction levels of ubiquinone.

Occurrence of ubiquinone-9 in the lung worm mitochondria has been previously demonstrated by the authors (Sato & Ozawa, 1969). In order to get information that ubiquinone is a functional component of the respiratory chain, the aerobic steady state oxidation-reduction levels of endogenous ubiquinone were determined.

Table 2 Steady state oxidation-reduction levels of ubiquinone in *Metastrongylus elongatus*

Substrate (25 mM)	Inhibitor	% reduction of mitochondrial ubiquinone (case)
—	—	0 (5)
succinate	—	46 (3)
succinate	antimycin A 10~26μg	49 (4)
succinate	KCN 5×10^{-3} M	75 (3)

Determinations were carried out with mitochondrial suspension as described in the Materials and Methods section.

When lung worm mitochondria were incubated with succinate as substrate under anaerobic condition the ubiquinone was reduced to ubiquinol. On aeration of the mixture the ubiquinol was oxidized back to ubiquinone. As shown in Table 2, the steady state concentration of the endogenous ubiquinol under aerobic condition accounted for about 45 % of the total extractable ubi quinone. In the presence of cyanide the level of ubiquinol raised and more than 70 % of the quinone could be isolated in its reduced from. Antimycin A, however, affected little the ratio.

Discussion

The present spectrophotometric examinations provided the evidence that an electron transport with cytochromes and ubiquinone is operative in the lung worm mitochondria.

In substrate reduced minus oxidized difference spectrum, a maximum corresponding to cytochrome a appeared at somewhat shorter wavelengths, 600 mμ, than that of a typical one, at 605 mμ. This coincided with the previous result obtained by the direct spectroscopic examination of lung worm mitochondria (Ozawa & Sato, 1966). Existence of a type a cytochrome was clearly demonstrated by the absorption maximum at 442 mμ in substrate reduced minus oxidized difference spectrum as well as a trough at 443 mμ in the substrate reduced+CO minus reduced difference spectrum. However, in the CO spectrum a typical absorption peak of CO bound cytochrome a_3 at 430 mμ could not be demonstrated. When CO was bubbled into a measure sample where cytochrome a and c were kept oxidized in the presence of antimycin A, a small absorption peak appeared at around 420 mμ as well. It has been already described by Chance (1957) that the effect of hemoglobin upon cytochrome a_3 assay leads to the result that the peak at 430 mμ of CO bound cytochrome a_3 is shifted to shorter wavelengths while trough at 445 mμ is very little affected. Therefore, it is probable that a peak of CO bound cytochrome a_3 shifted to the shorter wavelengths by interference of a hemoglobin like substance which might be unaffected by $NaNO_2$ treatment. Whether this CO-binding pigment was orginated solely from a host animal or was an unknown component of terminal oxidation reaction in lung worm mitochondria remains to be settled.

The absorption peaks of cytochrome b were clearly demonstrated in substrate reduced mitochondria treated with antimycin A minus oxidized difference spectrum. This as well as the inhibition of succinate cytochrome c reductase by antimycin A indicates that the site of inhibition of antimycin A lies between cytochrome b and c (c_1+c) as in mammalian respiratory chain.

On the other hand, NADH-cytochrome c reductase was insensitive to antimycin A. In acceptor controlled mitochondria oxidation of NAD-linked substrate was sensitive to antimycin A, while NADH added exogenously was scarsely used as substrate supposedly due to its impermeability (Ozawa & Sato, 1965). Therefore, this antimycin A insensitive reduction of cytochrome c occured probably in extra mitochondrial route as demonstrated in certain mammalian systems (Vernon et al., 1952; Devlin & Lehninger, 1956; Raw & Mahler, 1959). In fact, when NAD-linked substrate such as malate (+pyruvate) was used, the reduction of cytochrome c was sensitive to antimycin A.

Ubiquinones are an important family of lipid soluble benzoquinone which appears to play a major role in the electron transport

systems of respiration and photosynthesis (Morton, 1965). Although there are various views on its precise position in electron transfer sequence, it is generally held that ubiquinone acts as a hydrogen carrier in the electron transport system by virtue of its property of reversible oxidation-reduction (Green & Silman, 1967; Klingenberg & Kröger, 1967). In the present experiment no oxidation-reduction kinetics of endogenous ubiquinone was determined. Therefore no precise position of ubiquinone in electron transport chain could be assigned. However, the result from the steady state measurement of endogenous ubiquine supports the idea that ubiquinone acts as a hydrogen carrier in the lung worm mitochondria also.

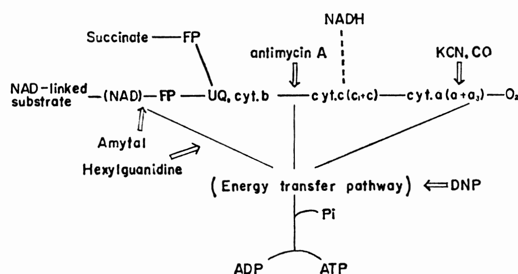

Fig. 6 A possible electron transport system in *Metastrongylus elongatus*.
———: Main pathway coupled with phosphorylation.
··········: Extra mitochondrial pathway.
Arrows indicate the the sites of inhibition by various inhibitors.

On the basis of the present spectrophotometric examinations of lung worm mitochondria, together with previously described inhibition studies on acceptor controlled lung worm mitochondria (Ozawa & Sato, 1965; 1966), a tentative scheme of a possible electron transport pathway in the lung worm mitochondria was presented (Fig. 6). Although an alternate pathway might be operative in nonphosphorylating mitochondria, as has been suggested by one of the authors (Ozawa & Fukushima, 1963), the scheme presented here shows the main electron transport pathway in phosphorylating lung worm mitochondria.

Summary

Spectrophotometric examinations of swine lung worm mitochondria were carried out, and the following results were obtained.

1. In succinate reduced minus oxidized difference spectrum absorption peaks of cytochrome a (600 mμ), b (560 mμ), c (550 mμ) and a composite consisting of these (peak; 442 mμ, shoulders; 430 mμ, 420 mμ) were revealed. In the presence of antimycin A absorption peaks of cytochrome b became prominant whereas those of cytochrome a and c disappeared.

2. In reduced+CO minus reduced difference spectrum, although a trough at 443 mμ of cytochrome a_3 appeared, a peak of CO-binding pigment shifted to rather shorter wavelengths (420 mμ) than that of a typical one (430 mμ).

3. Cytochrome oxidase activity was determined, which was inhibited by KCN (6.6×10^{-4} M).

4. NADH-cytochrome c reductase and succinate cytochrome c reductase activity were determined. While the latter was inhibited by animycin A (2.7 μg/ml), the former was insensitive to animycin A.

5. Steady state oxidation-reduction levels of endogenous ubiquinone were determined. In the presence of succinate about 45 % of the total extractable quinone were in reduced form. KCN increased the ratio of ubiquinol up to 75 %, while antimycin A did not alter the ratio significantly.

On the basis of these findings, together with previous evidences on the acceptor controlled lung worm mitochondria, a scheme of possible electron transport system was proposed.

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ブタ肺虫ミトコンドリアの電子伝達系

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ブタ肺虫ミトコンドリアの電子伝達因子について知るため，肺虫ミトコンドリアの分光測光学的検索をし下記の諸結果を得た．

1. コハク酸を基質としてのミトコンドリアの酸化-還元差スペクトルで，チトクロム a, b, c にそれぞれ相当する 600 mμ, 560 mμ, 550 mμ の吸収極大およびソーレット帯における 442 mμ の吸収極大，430 mμ, 420 mμ の肩が観察された．antimycin A の存在下ではチトクロム a, c の吸収極大は消失し，チトクロム b の吸収極大が顕著になつた．

2. 予め還元型に保つたミトコンドリアの CO 差スペクトルで，チトクロム a_3 に相当する 443 mμ の吸収の谷が現われたが，CO 結合 a_3 の吸収極大は典型的な 430 mμ よりもやや低波長側にづれた 420 mμ に現われた．

3. チトクロム c 酸化酵素活性を肺虫ミトコンドリアについて測定したが，その比活性は 1.47 μmole チトクロム c 酸化/hr. mg タンパク量であり，本酵素はKCNにより阻害される．

4. NADH チトクロム c 還元酵素ならびにコハク酸チトクロム c 還元酵素の存在が認められたが，後者は antimycin A (2.7 μg/ml) で阻害されるが前者は antimycin A 不感受性であつた．

5. 肺虫ミトコンドリアの内在ユビキノンの酸化還元に対する基質，阻害剤の影響を調べたが，コハク酸存在下約 45 % が還元型ユビキノンとして存在するが，KCN によりその割合が 75 % に増加した．antimycin A はその割合を有意に変化させなかつた．

これらの実験結果は肺虫ミトコンドリアの電子伝達系がチトクロム a, b, c およびユビキノンを電子伝達因子としてもつことを示しており，これまで呼吸調節能のある肺虫ミトコンドリアについて得た実験事実と合わせ，その電子伝達の推定経路について考察を加えた．