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Developmental changes in the respiratory chain of *Ascaris* mitochondria

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The *Ascaris* larval respiratory chain, particularly complex II (succinate-ubiquinone oxidoreductase), was characterized in isolated mitochondria. Low-temperature difference spectra showed the presence of substrate-reducible cytochromes aa_3 of complex IV, $c + c_1$ and b of complex III (ubiquinol-cytochrome c oxidoreductase) in mitochondria from second-stage larvae (L2 mitochondria). Quinone analysis by high-performance liquid chromatography showed that, unlike adult mitochondria, which contain only rhodoquinone-9, L2 mitochondria contain ubiquinone-9 as a major component. Complex II in L2 mitochondria was kinetically different from that in adult mitochondria. The individual oxidoreductase activities comprising succinate oxidase, and fumarate reductase were determined in mitochondria from L2 larvae, from larvae cultured to later stages, and from adult nematodes. The L2 mitochondria exhibited the highest specific activity of cytochrome c oxidase, indicating that L2 larvae have the most aerobic respiratory chain among the stages studied. The Cyb_S subunit of complex II in L2 and cultured-larvae mitochondria exhibited different reactivities against anti-adult Cyb_S antibodies. Taken together, these results indicate that the complex II of larvae is different from its adult counterpart. In parallel with this change in mitochondrial biogenesis, biosynthetic conversion of quinones occurs during development in *Ascaris* nematodes.

Introduction

Adults of the parasitic nematode of swine, *Ascaris suum*, inhabit the microaerobic lumen of the small intestine. They have an anaerobic-energy metabolism where, instead of complete oxidation of glucose, a variety of reduced compounds are produced as end-products of carbohydrate metabolism. These compounds are succinate and volatile organic acids including acetate, propionate, 2-methylvalerate and 2-methylbutyrate [1,2]. Among metabolic pathways producing these compounds, the series of reactions leading to succinate is designated as the PEPCK (phospho*enol*-pyruvate carboxykinase)-succinate pathway, referring to the key enzyme in the cytosol and to the end-product [3]. The final reaction of this pathway is catalyzed by the mitochondrial NADH-fumarate reductase system which catalyzes the anaerobic reduction of fumarate to succinate and is coupled to ATP synthesis [4–6]. The NADH-fumarate reductase system consists of respiratory-chain complexes I (NADH-ubiquinone oxidoreductase) and II (succinate-ubiquinone oxidoreductase) and rhodoquinone. Electrons from NADH are accepted by complex I, used to produce rhodoquinol, and then transferred to fumarate through complex II acting in its reverse direction (fumarate reduction) [7–9]. It should be stressed that, instead of ubiquinone, which is present in mammalian mitochondria, adult *Ascaris* mitochondria contain rhodoquinone, which as a low-potential electron carrier is indispensable for the NADH-fumarate reductase system [9]. Also, unlike the bovine heart enzyme, complex II from adult *Ascaris* mitochondria exhibits high fumarate reductase (FRD) activity over succinate dehydrogenase (SDH) activity [9] as well as unique proper-

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Abbreviations: PEPCK, phospho*enol*pyruvate carboxykinase; FRD, fumarate reductase; SDH, succinate dehydrogenase; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine; TTFA, 2-thenoyltrifluoroacetone; Fp, flavoprotein subunit of complex II; Ip, iron-sulfur protein subunit of complex II; Cyb_L and Cyb_S, large and small subunit of cytochrome *b*-558 of complex II, respectively. E'_m, oxidation-reduction midpoint potential at neutral pH.

ties in its redox centers [10,11]. Furthermore, mitochondria from adult *Ascaris* have reduced or low levels of complex III (ubiquinol–cytochrome *c* oxidoreductase), cytochrome *c*, and complex IV (cytochrome *c* oxidase) [5,7], thereby resulting in a relatively high content of complex II [12]. Thus, mitochondria from adult *Ascaris* function anaerobically, a physiological adaptation to their microaerobic habitat [3,13].

In contrast to adult nematodes, the fertilized eggs require oxygen for embryonation and development to second stage larvae (L2s), whose energy-metabolism is aerobic with a functional cytochrome *c* oxidase [14]. When infective eggs containing L2s are swallowed by the host, they hatch in the intestine and the L2s migrate first to the liver. There they molt to third-stage larvae (L3s) before further migration to the lungs. From the lungs, the L3s migrate back to the small intestine where they undergo the third and fourth molts, developing into fourth stage larvae (L4s) and young adults, respectively [15]. Thus, during its complex life cycle, the *Ascaris* nematode encounters a fluctuating environment in which such factors as p_{O_2}, p_{CO_2}, redox potential, and pH, change depending on the organs or tissues to which they migrate.

Evidently, during development from L2 to adult, a transition from aerobic to anaerobic metabolism takes place [13]; L3s appear to depend predominantly on aerobic metabolism, although they exhibit elevated activities of enzymes typical of anaerobic metabolism, including PEPCK and malic enzyme [16, 17]. Upon further development to L4s, the larvae become capable of synthesizing end-products typical of the adult and they become cyanide-insensitive in terms of motility, although they still retain cytochrome *c* oxidase activity. The metabolic transition from aerobic to anaerobic is expressed as a respiratory transition at the mitochondrial level, but the underlying molecular and genetic mechanisms are not yet known [18]. In contrast to the situation with the anaerobic respiratory chain of adult *Ascaris* mitochondria, quantitative information on individual electron transfer components, and on complex II in particular, is limited for the larval stages [19–21]. The present communication deals with the *Ascaris* larval respiratory chain and its developmental changes. Mitochondria isolated from in vitro cultured larvae are characterized by spectrophotometry, by immunoblotting using anti-adult complex II antibodies, and by analysis of quinone content.

Materials and Methods

Fertilized eggs and second stage larvae (L2s) were prepared according to the methods of Costello [22] and Urban et al. [23], respectively. To obtain larvae at further stages of development, second stage larvae were cultivated continuously in a three step roller culture system according to the method of Douvres and Urban [24]. The larvae were harvested after 17 days in culture (17 DIC) and 35 DIC. Stages of development were based on morphological criteria for L3 and L4 [15]; 17 DIC and 35 DIC larvae were shown to be L3 and a mixture of L3 (31%) and L4 (69%), respectively.

Adult *Ascaris* mitochondria were prepared as previously described except that Nagase treatment was omitted [7,12]. Mitochondria were also prepared from eggs [9], larvae [21], rat liver and heart [25], and bovine heart [26] according to established methods. Submitochondrial particles were prepared as previously reported [7].

Measurement of enzymatic activities. Fumarate reductase activity was determined at 25°C according to the reported method [27] except that reduced methyl viologen was used as an electron donor. NADH-fumarate reductase activity was determined by monitoring the oxidation of NADH at 340 nm as described [9]. Cytochrome oxidase activity was measured at 25°C as described [7], except that 25 mM potassium phosphate buffer (pH 7.4) containing 1% (v/v) Tween 80 was used as the reaction buffer. Succinate-ubiquinone-1 and succinate-cytochrome *c* reductases were assayed at 25°C as described [7,8].

Spectrophotometry. Low-temperature difference spectra were recorded on a dual-wavelength spectrophotometer (model DW-2, American Instrument Co., Silver Spring, MD); the concentrations of cytochromes in larval mitochondria were determined from reduced-minus-oxidized difference spectra as previously reported [7]. The extinction coefficients used for cytochromes *b*, $c + c_1$, and aa_3 were $\Delta\epsilon_{562-575} = 17.9$ mM^{-1} cm^{-1}, $\Delta\epsilon_{550-540} = 19.0$ mM^{-1} cm^{-1} and $\Delta\epsilon_{605-630}$ mM^{-1} cm^{-1}, respectively, and an intensification factor of 7 was used for all the cytochromes to calculate contents from low-temperature spectra [7].

Extraction and determination of quinones in Ascaris *mitochondria.* Mitochondrial quinones were extracted according to the method of Szarkowska [28] with some modifications. Mitochondria from *Ascaris* eggs, larvae, and adult worms (3–12 mg of mitochondrial protein) were lyophilized in a glass-stoppered tube. The lyophilized mitochondria were dispersed in 2 ml of n-pentane. The suspension was then mixed vigorously on a Vortex mixer for 10 min and centrifuged at $750 \times g$ for 5 min. The supernatant was removed and retained. The extraction was repeated four times in the same manner and the resulting extracts were pooled. The combined extract was dried under a stream of argon gas and the residue was redissolved in 2 ml of ethanol.

An aliquot of the ethanolic solution was analyzed by HPLC according to the method of Hiraishi [29]. HPLC was carried out on a Shimadzu Liquid Chromotograph LC-3A equipped with a Zorbax ODS pre-packed col-

umn (4.6 × 250 mm) as the main column, a Permophase ODS column (4.6 × 50 mm) as the pre-column, a UV spectrophotometric detector (Shimadzu SPD-6A), and a microcomputor (Shimadzu Chromatopack C-RIA) for calculating the peak areas. The quinones were eluted with methanol-isopropyl ethers (4:1, v/v) at a flow rate of 1 ml/min. The eluted quinones were identified by comparing their retention times with a mixture of authentic quinones (rhodoquinone-9 and ubiquinones-5, 6, 7, 8, 9, 10) and their concentrations were determined from calibration curves. Concentrations of standard rhodoquinone-9 and ubiquinone-9 were determined spectrophotometrically using coefficents of $E_{1\%1cm}$ at 283 nm = 141 [30] and of $E_{1\%1cm}$ at 275 nm = 158 [31], respectively. The quinones were also analyzed by reverse-phase TLC (Whatman LKC18F, 200 μm) with acetone/water (95:5%) as the running solvent. Following development, the separated quinones were detected with a UV lamp and the appropriate areas of the chromotogram were scraped into a glass-stoppered tube. The quinones were extracted with 0.2 ml ethanol and their spectra were recorded on a spectrophotometer (Shimadzu UV-265FW) using microcuvettes.

SDS-polyacrylamide gel electrophoresis and immunoblotting. SDS-polyacrylamide gel electrophoresis was performed as previously described [32]. Antibodies against *Ascaris* complex II subunits were prepared as described [9]. Immunoblotting of mitochondria was performed according to the method of Towbin [33] using alkaline phosphatase-conjugated anti-antibody [34]. For quantitative analyses, different concentrations of purified adult complex II were also subjected to immunoblotting at the same time, and the developed sheets were scanned through a densitometer (Shimadzu Dual-wavelength flying-spot scanner CS-9000) to calculate the relative densities by normalization with the protein contents.

Protein was determined by the Hartree modification of the Lowry method with bovine serum albumin as the standard [35]. Ubiquinone analogues with various numbers of isoprenoid chains were kindly provided by Eisai

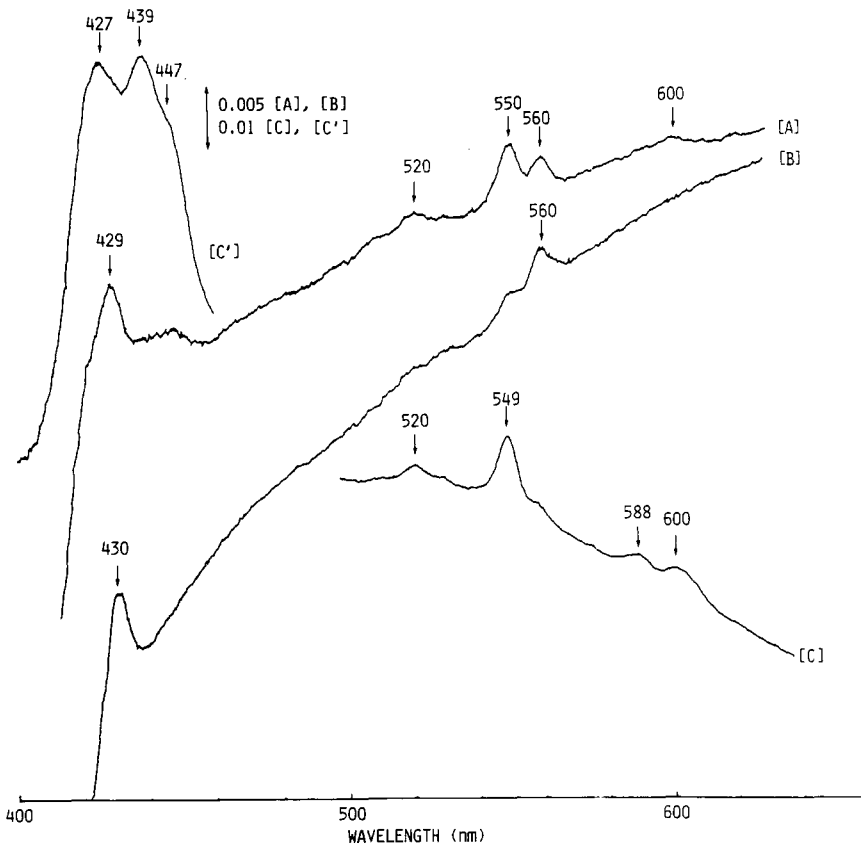

Fig. 1. Low-temperature difference spectra of L2 mitochondria reduced with various substrates. The reaction medium consisted of 3.3 mM Tris-HCl buffer (pH 7.4), 50% (v/v) glycerol, 83 mM sucrose, and 0.06 mM EDTA. L2 mitochondria, suspended in the reaction medium, were flushed with oxygen gas for 2 min and used as the oxidized sample. To make reduced samples, the oxidized mitochondria were incubated with various substrates for 5 min at room temperature and frozen in liquid nitrogen. Trace A. The spectrum of mitochondria reduced with 10 mM potassium succinate in the presence of 1 mM KCN minus oxidized mitochondria. Trace B. The spectrum of mitochondria reduced with 10 mM potassium succinate in the presence of 3.6 μM antimycin A minus oxidized mitochondria. Trace C. The spectrum of mitochondria reduced with 10 mM ascorbate and 0.2 mM TMPD in the presence of 1 mM KCN minus oxidized mitochondria. The protein concentration was 1.35 mg/ml. The cuvette volume was 0.5 ml and the light path was 2 mm.

TABLE I

Comparison of cytochrome contents in mitochondria from Ascaris and bovine heart

n.d., not detected.

Source	nmol/mg protein			*b*:	$c + c_1$:	aa_3
	b	$c + c_1$	aa_3			
Ascaris L2	0.44	0.37	0.15	1	0.83	0.34
Ascaris adult [a]	0.19	0.036	n.d.	1	0.19	–
Bovine heart [b]	0.32	0.47	0.68	1	1.5	2.1

[a] From results reported by Takamiya et al. [7].
[b] From results reported by Merle and Kadenbach [37].

Co., Tokyo. All other chemicals were of analytical grade.

Results

Cytochrome components in L2 mitochondria

Cytochrome components in isolated mitochondria from L2 larvae were studied by spectrophotometry at low temperature. Upon reduction with succinate in the presence of KCN, three α-peaks at 550, 560 and 600 nm, corresponding to cytochromes $c + c_1$, *b* and aa_3, respectively, and a γ-peak at 429 nm was observed in the difference spectrum (Fig. 1A). The γ-peak at 429 nm and the α-peak at 560 nm are derived from the type *b* cytochrome associated with complex III, not from the cytochrome *b* in complex II, since the latter has γ- and α-bands at shorter wavelengths (γ-band at 424 nm and α-band at 558 nm for complex II cytochrome *b*) [8]. This was confirmed by observing the spectrum produced in the presence of antimycin A, an inhibitor that blocks electron flow between cytochromes *b* and c_1 associated with complex III; a sharp γ-band at 430 nm and an intensified α-peak at 560 nm appeared in the spectrum (Fig. 1B). Thus, the cytochrome *b* of complex II is not detectable in L2 mitochondria after succinate reduction. These findings are in sharp contrast to those obtained with adult mitochondria [7] where succinate reduction results in predominant appearance of cytochrome *b* of complex II. These results indicate that in L2 mitochondria, the ratio of complex III to complex II is much greater than in adult mitochondria.

The occurrence of cytochromes $c + c_1$ and aa_3 in L2 mitochondria was verified using ascorbate and TMPD as reductants; the spectrum shows a distinct α-peak at 549 nm and a γ-peak at 427 nm derived from cytochromes $c + c_1$, and two α-peaks at 588 and 600 nm and a γ-peak at 439 nm, which are derived from cytochrome aa_3 (Fig. 1C) [36]. Table I shows the cytochrome contents and their relative ratios in L2 mitochondria compared with the data reported for adult *Ascaris* [7] and bovine heart [37] mitochondria. The content of cytochrome $c + c_1$ is 10-times higher in L2 mitochondria than in adult mitochondria. Futhermore, high concentrations of cytochrome aa_3 occur in L2 mitochondria, whereas it is barely detectable in adult mitochondria. These spectral data show that the cytochrome components required for aerobic metabolism,

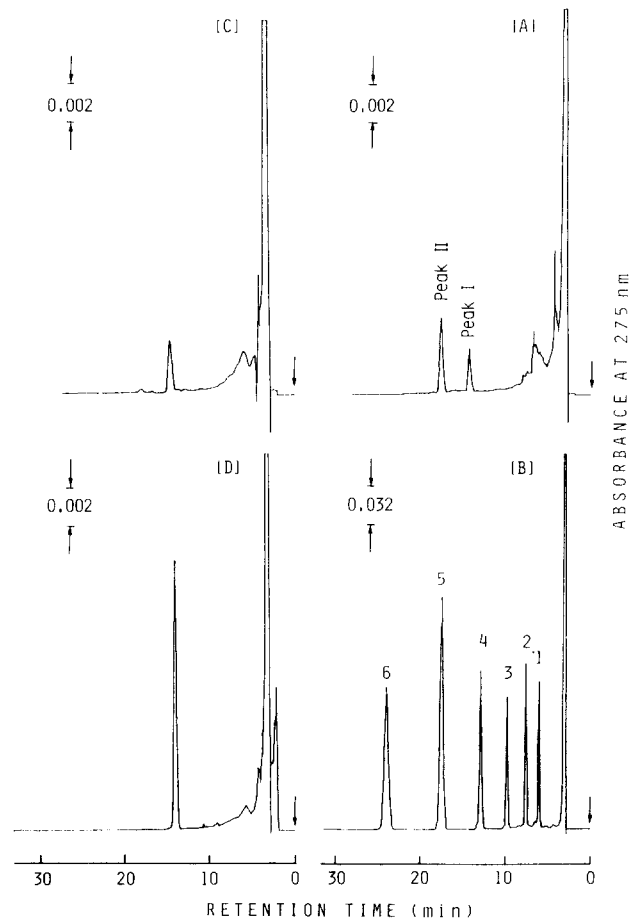

Fig. 2. HPLC of quinones extracted from *Ascaris suum* mitochondria at various stages of development. Detailed experimental conditions are described in Materials and Methods. The arrows indicate sample injection. (A) L2 mitochondria. (B) Authentic ubiquinones with various isoprenoid chain lengths. 1: ubiquinone-5, 2: ubiquinone-6, 3: ubiquinone-7, 4: ubiquinone-8, 5: ubiquinone-9, 6: ubiquinone-10. (C) Mitochondria from unembryonated eggs. (D) Adult muscle mitochondria.

and, consequently, electron-transfer complexes III and IV, are enriched in larval mitochondria, specifically at the L2 stage.

Quinone components in L2 mitochondria

To determine whether the reactions catalyzed by complex II in L2 mitochondria involve net succinate oxidation or fumarate reduction, it is necessary to examine the mitochondrial quinones that react physiologically with complex II. Quinones were extracted from the mitochondria of L2 larvae, from unembryonated eggs, and from adult *Ascaris* muscle and analyzed by HPLC. As shown in Fig. 2, L2 mitochondria contain two quinone components (Peaks I and II), whereas egg mitochondria contain, essentially, one component whose retention time is the same as that for Peak I (Fig. 2A, C). The retention time of Peak I is the same as that of rhodoquinone-9 extracted from adult *Ascaris* muscle (Fig. 2D). On the other hand, the Peak II component elutes at a retention time corresponding to that for authentic ubiquinone-9 (Fig. 2A, B).

After repeated HPLC separations, Peaks I and II were further purified by preparative TLC and their absorption spectra were recorded. As can be seen in Fig. 3A, the Peak II component has an absorption maximum at 275 nm. Upon reduction with $NaBH_4$, the absorption was reduced and the maximum shifted to 290 nm. In the case of Peak I, the extracted material exhibits an absorption maximum at 280 nm and a broad absorption around 500 nm (Fig. 3B). When reduced with $NaBH_4$, the 280 nm absorption was reduced and the broad absorption around 500 nm completely disappeared. These spectral data indicate that

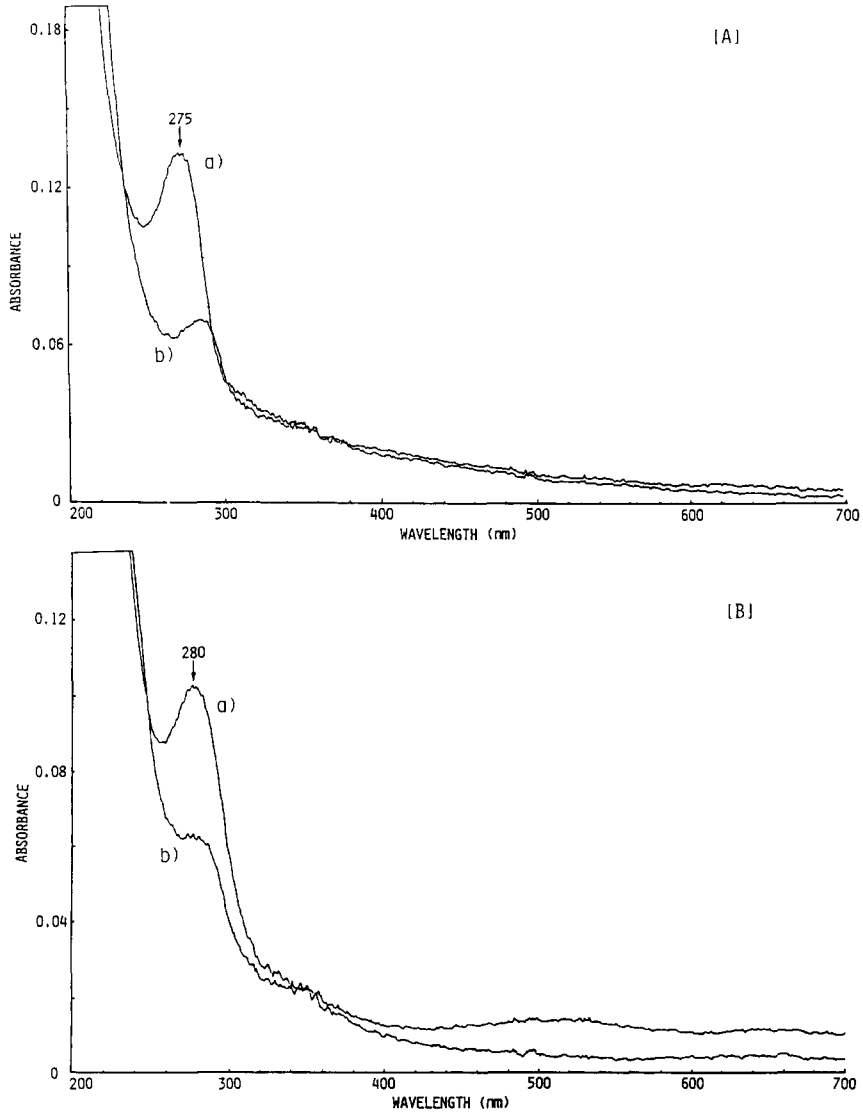

Fig. 3. Absorption spectra of quinones isolated from L2 mitochondria. Detailed experimental conditions are described in Materials and Methods. (A) Peak II, (a) non-treated, (b) reduced with $NaBH_4$. (B) Peak I, (a) non-treated, (b) reduced with $NaBH_4$. Light path of the microcuvette was 1 cm.

TABLE II

Quinone content of Ascaris mitochondria

Content in nmol/mg protein. n.d., not detected.

Stage	Ubiquinone-9	Rhodoquinone-9
Egg	n.d.	0.0054
L2	0.33	0.12
Adult	n.d.	1.9

the Peak I and II components are rhodoquinone and ubiquinone, respectively; the number of isoprenoid side chains in both quinones was found to be 9 by HPLC analysis. As shown in Table II, L2 mitochondria contain ubiquinone-9 as a major component (73.3% of the total quinone content) with rhodoquinone-9 accounting for the remainder (26.7%). In contrast, mitochondria from unembryonated eggs and adult *Ascaris* contain only rhodoquinone-9, confirming the previous data by Sato and Ozawa [38]. The low concentration of rhodoquinone-9 in the unembryonated egg mitochondria may reflect their low respiratory activity [14].

Properties of L2 complex II

The K_m for succinate in succinate dehydrogenation and that for fumarate in NADH-linked fumarate reduction were determined in mitochondria from *Ascaris* L2 larvae and adults and from bovine heart. As shown in Table III, in adult *Ascaris* mitochondria, the apparent K_m for fumarate (0.031 mM) is about 1/24th that for succinate (0.74 mM), suggesting that at low substrate concentrations the reaction will operate in the direction of fumarate reduction. Higher K_m values for fumarate of the isolated complex II and of adult *Ascaris* mitochondria (3.09 and 3.3 mM, respectively) were reported in a previous study [9]. The descrepancy may be due to the different assay method; in the previous study, reduced methyl viologen was used as an artificial electron donor, whereas NADH was employed in the present study. Interestingly, a low K_m value for fumarate (0.027 mM) has also been observed for mitochondria from *Tubifex* sp., a fresh water facultative anaerobe [39] and from *Paragonimus westermani*, a lung fluke (unpublished data). In contrast, the

TABLE III

Comparison of K_m values for succinate-fumarate conversion in mitochondria from Ascaris and bovine heart

Source	K_m (mM)	
	succinate	fumarate
Ascaris L2	0.19	0.15
Ascaris adult	0.74	0.031
Bovine heart	0.32	0.21

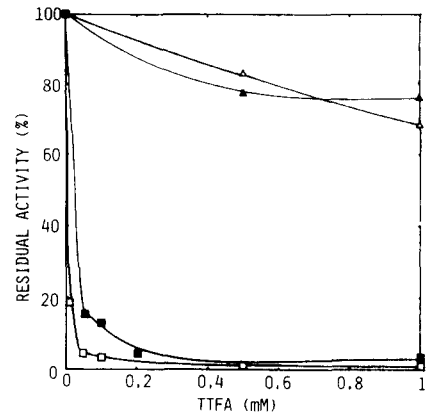

Fig. 4. Effect of TTFA on succinate-cytochrome *c* reductase in mitochondria from various sources. △, *Ascaris* adult; ▲, *Ascaris* L2; ■, rat liver; □, rat heart. The specific activities in the absence of TTFA were as follows: 0.03 μmol/min per mg for *Ascaris* adult; 0.09 μmol/min per mg for L2; 0.13 μmol/min per mg for rat liver; 0.20 μmol/min per mg for rat heart. In this experiment, cytochrome *c* was used instead of ubiquinone-1 as the electron acceptor because TTFA has an absorption around 275 nm that severely disturbs activity measurement using ubiquinone-1.

K_m values for succinate and fumarate are much closer in L2 mitochondria (Table III). A high K_m value for fumarate was also obtained in bovine heart mitochondria. These results are similar to those of King and Takemori [40], who reported values of 0.28 and 0.38 mM for the K_m for succinate and the K_i for fumarate, respectively, in their study of bovine heart succinate-cytochrome *c* reductase. Therefore, the pathway responsible for succinate-fumarate conversion (complex II) in *Ascaris* larval tissues differs from that in mitochondria of adults in its kinetic properties. As with bovine heart complex II, the complex in larvae appears to function less efficiently as a fumarate reductase.

The succinate-ubiquinone reductase and fumarate reductase activities of adult *Ascaris* complex II are insensitive to TTFA (2-thenoyltrifluoroacetone), a potent inhibitor of complex II in aerobic tissues such as bovine heart [9,41]. Thus, the effects of TTFA on succinate dehydrogenase activity were compared for mitochondria from adult and larval *Ascaris* and mammalian tissues. As shown in Fig. 4, L2 mitochondria are as insensitive to TTFA as adult mitochondria, whereas mammalian mitochondria are highly sensitive. Since TTFA inhibits the re-oxidation of the iron-sulfur center (S-3) of bovine complex II [42,43], which is associated with the Ip subunit, the insensitivity of *Ascaris* complex II toward TTFA may indicate a different pathway for electron transfer in the Ip subunit. Alternatively, it may reflect structural difference in the vicinitiy of the S-3 center, which results in different accessibility of TTFA.

TABLE IV

Oxidoreductase activities of Ascaris mitochondria at various stages

UQ_1, ubiquinone-1.

Stage	Succinate-UQ_1 (μmol min^{-1} mg^{-1})	Succinate-cyt *c* (μmol min^{-1} mg^{-1})	Cyt *c* oxidase (s^{-1} ml^{-1} mg^{-1})	Fumarate reductase (μmol min^{-1} mg^{-1})	SDH/FRD
L2	0.065	0.10	0.35	0.074	0.87
17 DIC	0.064	0.15	0.15	0.22	0.29
35 DIC	0.118	0.095	0.091	0.63	0.18
Adult	0.136 [a]	0.038	0.011	2.72 [a]	0.05

[a] From the results reported by Kita et al. [9].

Developmental changes in respiratory enzyme activities and properties of complex II

To further define the transition from aerobic to anaerobic metabolism, individual oxidoreductase activities and subunit compositions of complex II were assayed in mitochondria isolated from L2s, cultured larvae at further stages of development, and adult nematodes. As shown in Table IV, the specific activity of cytochrome *c* oxidase was more than 30-times greater in L2 mitochondria than in adult mitochondria. As larvae develop further from L2, the specific activity of cytochrome *c* oxidase decreases, whereas that of fumarate reductase (FRD) increases remarkably. In contrast, the level of succinate-ubiquinone-1 reductase (SDH) appears to be relatively constant, except in the 35 DIC larvae. Consequently, the highest SDH/FRD ratio (0.87) was obtained for L2 larvae. These data further confirm that L2 larvae have the most active aerobic metabolism of the developmental stages.

Complex II from adult *Ascaris* muscle was previously shown to consist of four different subunits: a flavoprotein subunit (Fp, M_r = 68 000) containing flavin as a prosthetic group [44], an iron-sulfur protein subunit (Ip, M_r = 26 000) associated with iron-sulfur clusters [9], and two small subunits (Cyb_L and Cyb_S, M_r = 15 000 and 13 500, respectively) ligating heme *b* to form cytochrome *b*-558 [9]. To investigate the subunit composition of *Ascaris* complex II at various developmen-

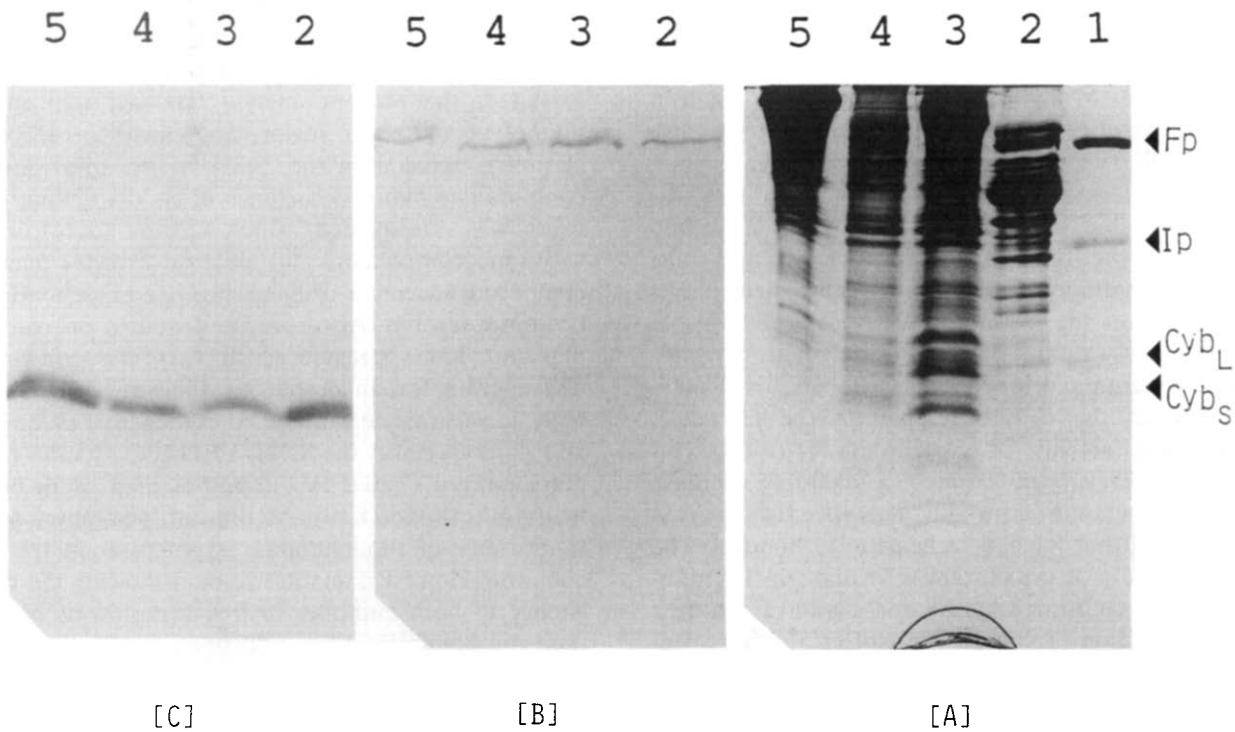

Fig. 5. Immunoblotting of larval and adult *Ascaris* mitochondria. (A) The SDS-polyacrylamide gel stained for protein. Lane 1, purified adult complex II (10 μg); lane 2, adult muscle mitochondria (42.6 μg); lane 3, L2 mitochondria (46 μg); lane 4, mitochondria from 17 DIC larvae (39.6 μg); lane 5, mitochondria from 35 DIC larvae (42.4 μg) (B) Immunoblot with anti-adult *Ascaris* Fp subunit. lane 2: adult mitochondria; lane 3: L2 mitochondria; lane 4: mitochondria from 17 DIC larvae; lane 5: mitochondria from 35 DIC larvae. The amounts of mitochondria loaded were the same as described in (A). (C) Immunoblot with anti-adult *Ascaris* Cyb_S subunit. Lane 2, adult mitochondria; lane 3, L2 mitochondria; lane 4, mitochondria from 17 DIC larvae; lane 5, mitochondria from 35 DIC larvae. The amounts of mitochondria loaded were the same as described in (A).

tal stages, the mitochondria from larvae and adults were immunoblotted with anti-adult *Ascaris* complex II antibodies (Fig. 5). In this experiment, both anti-Fp and anti-Cyb_S antibodies were used. When anti-Fp antibody was used, crossreacting materials were observed for all mitochondria at a position corresponding to the Fp subunit of purified adult complex II (Fig. 5A, B). The ratios to the adult of the larval Fp subunit are as follows; adult : L2 : 17DIC : 35DIC = 1 : 1.47: 1.09 : 1.07.

In the case of the anti-Cyb_S subunit antibody, crossreacting materials were also observed in all mitochondria at a position corresponding to the Cyb_S subunit of complex II (Fig. 5A, C). The intensity of color development of this band appears to increase along with larval development. The approximate ratios varied among the developmental stages in the following order: adult : L2 : 17DIC : 35DIC = 1 : 0.42 : 0.70 : 1.40. These results suggest that the reactivity with the anti-Cyb_S subunit antibody differs among the developmental stages or, alternatively, that the specific content of subunit Cyb_S increases with larval development. Interestingly, the specific activity of fumarate reductase appears to correlate with the relative density ratios of the Cyb_S subunit.

Discussion

The present data show L2 to be the most aerobic among the *Ascaris* developmental stages both in terms of cytochromes (Table I) and the individual electron transport activities comprising the succinate-oxidase system (Table IV). Using homogenates of *Ascaris* eggs, Oya et al. determined that cytochrome *c* oxidase was barely detectable in unembryonated eggs, increased markedly at the time of molting into second-stage larvae, and continued to rise during subsequent development [14]. In the present study, very low levels of cytochrome *c* oxidase were also detected in mitochondria from undeveloped eggs ($1.81 \cdot 10^{-4}$ s^{-1} ml^{-1} mg^{-1}). When the eggs developed to the L2 stage, however, the activity of cytochrome *c* oxidase increased to 0.35 s^{-1} ml^{-1} mg^{-1}, a 2000-fold increase based on mitochondrial protein. This specific activity is about 70% of that for bovine heart mitochondria. The elevated activity of cytochrome *c* oxidase in L2 mitochondria was confirmed by the analysis of cytochrome aa_3 concentration (Table I). Hayashi et al., in a spectrophotometric study, also reported an increase in the amount of cytochrome aa_3 in 1st and 2nd stage larval homogenates [45].

Adult *Ascaris* mitochondria, unlike mammalian mitochondria, are known to contain rhodoquinone only [38]. Rhodoquinone is the derivative of ubiquinone in which one of methoxy groups is replaced by an amino group (Fig. 6). The oxidation-reduction midpoint potential (E'_m) of rhodoquinone is much lower than that of ubiquinone. Our previous data showed that rhodoquinone is required for reconstituting the NADH-fumarate reductase system with bovine heart complex I and *Ascaris* complex II; in this case, rhodoquinone functions as a low-potential electron transfer component of the reductase system [9]. Only ubiquinone occurs in mammalian mitochondria, where it serves as a high redox potential electron carrier in both the succinate- and NADH-oxidase systems. As expected, the present analyses showed that ubiquinone-9 occurs as a major component in L2 mitochondria, accounting for 73.3% of the total quinone content. The minor occurrence of rhodoquinone, however, was unexpected, since current data on cytochrome components and electron transfer activities composing succinate oxidase show L2 mitochondria to be quite aerobic. Also, previous studies on oxidative phosphorylation stressed aerobic aspects, with no evidence given for anaerobic metabolism [21]. Interestingly, in accordance with the co-existence of ubiquinone and rhodoquinone, the SDH/FRD activity ratio for L2 mitochondria (Table IV) is not as high as in bovine heart mitochondria [9]. At present, the physiological significance of rhodoquinone is not clear in L2 mitochondria. However, qualitative data showing the co-existence of both quinones in free-living forms of other parasitic helminths have also been reported by Allen [46].

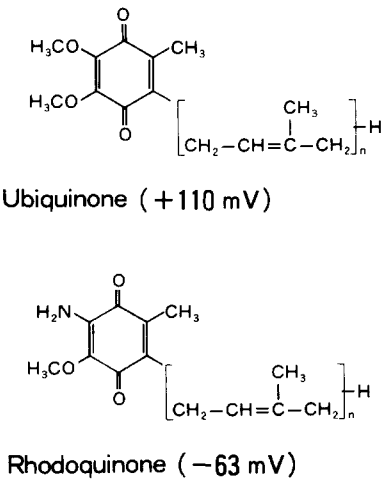

Fig. 6. Chemical structures and E_m values of ubiquinone and rhodoquinone. *n*, numbers of isoprenyl groups in side-chain.

When L2 larvae developed to the L3 and L4 stages under the cultivating conditions used, the specific activity of fumarate reductase increased remarkably [47]. In contrast to fumarate reductase, the level of cytochrome *c* oxidase decreased with larval development. However, 35 DIC (L3, 31%; L4, 69%) larval mitochondria still retain about one-fourth of the cytochrome *c* oxidase

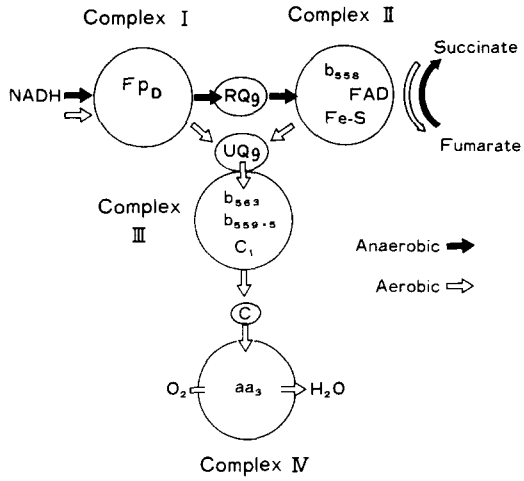

Fig. 7. Aerobic and anaerobic respiratory chains functioning in L2 and adult *Ascaris* mitochondria. Fp_D, NADH dehydrogenase; FAD, flavin adenine dinucleotide; Fe-S, iron-sulfur centers; RQ_9, rhodoquinone-9; UQ_9, ubiquinone-9.

found in L2 mitochondria and about 20-fold more than in adult muscle mitochondria. These values are compatible with the recent data by Vanover-Dettling and Komuniecki [17], who found a high level of cytochrome *c* oxidase in homogenates of L3 larvae recovered from lung tissues, and in L4 larvae obtained by cultivating L3 larvae under the same gas phase as used in the current study (85% N_2, 5% O_2, 10% CO_2).

The present study also suggests the occurrence of larval and adult isoforms of complex II that are developmentally regulated during the life cycle of the *Ascaris* nematode. This conclusion is based upon two sets of observations. First, the different kinetic parameters found for succinate-fumarate conversion catalyzed by mitochondria from L2 larvae and adults. Second, the variation of the SDH/FRD ratio that occurs during development, and more directly, the marked changes observed in the reactivity against anti-adult Cyb_s antibody.

In *Ascaris* nematodes, the developmental transition from aerobic to anaerobic respiration takes place during the life cycle (Fig. 7). Thus, they adapt themselves to the fluctuating environments by modulating their respiratory chain. To elucidate mechanisms for the respiratory transition, further study on biogenesis of the aerobic and anaerobic mitochondria is required.

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