BBA 46193

OXIDATIVE PHOSPHORYLATION IN MONIEZIA MUSCLE MITOCHONDRIA*

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(Received May 10th, 1971)

SUMMARY

1. Mitochondria isolated from *Moniezia expansa*, an intestinal parasitic tapeworm, have the same structural characteristics as the classical mammalian aerobic mitochondria by having clearly defined outer and inner membranes, outer compartment and intracristal spaces, and elementary particles attached to the mitochondrial cristae.

2. Intact Moniezia mitochondria are impermeable to NADH. The oxidation of α-glycerophosphate, succinate and ascorbate *plus* tetramethyl-*p*-phenylenediamine (TMPD), L-malate, pyruvate *plus* malate and L-glutamate *plus* malate, L-glutamate and pyruvate (in decreasing order of activity) was stimulated by ADP. Succinate oxidation has a K_m of 28 μM ADP.

3. α-Glycerophosphate and succinate oxidation have an ADP/O ratio of 1.5. Oxidative phosphorylation in Moniezia was inhibited by the classical mammalian inhibitors, oligomycin and atractyloside, and the oligomycin inhibition could be relieved by the uncoupler, *p*-trifluoromethoxycarbonylcyanidephenylhydrazone (FCCP).

4. The oxidation of the NAD^+-linked substrate, pyruvate *plus* malate, was piericidin A sensitive. Complete inhibition of the State 3 rate was achieved at 50 pmoles piericidin A per mg mitochondrial protein.

5. 8.0 ng antimycin A (approx. 16 pmoles) per mg mitochondrial protein inhibited 50 % of the succinoxidase activity (State 3). 33 % of Moniezia mitochondrial respiration (State 3) with succinate as substrate was antimycin A insensitive from about 20 to 230 ng antimycin A per mg protein.

6. α-Glycerophosphate, succinate and cytochrome oxidase (EC1.9.3.1) activities were very sensitive to CN^-. The amount of CN^- required to give 50 % inhibition (K_i) was 8μM (without FCCP) and 24 μM (with FCCP) for succinate oxidation (State 3), and 20 μM (with FCCP) for both the α-glycerophosphate and cytochrome oxidase activities (State 3).

INTRODUCTION

Intestinal parasites living in an environment of low and fluctuating oxygen tension have been considered and accepted by various investigators[1–4] to be anaerobes

Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; FCCP *p*-trifluoromethoxycarbonylcyanidephenylhydrazone.

* A preliminary report was presented at the International Symposium on the Comparative Biochemistry of Parasites, Janssen Research Foundation, Beerse, Belgium, 1–3rd September, 1971.

but not without controversy[5]. Recently, there has been strong evidence supporting the argument that intestinal parasites could very well be aerobic. In *Ascaris lumbricoides*, a roundworm, the biosynthesis of hydroxyproline for collagen formation requires oxygen[6–7]. Carefully prepared intact Ascaris muscle mitochondria have a respiratory chain containing functional *b*-, *c*- and *a*-type cytochromes, and Ascaris mitochondrial respiration was also sensitive to CN^- and CO with cytochrome a_3 acting as the terminal oxidase[8]. Cytochrome a_3 was also spectrophotometrically identified at liquid N_2 temperature (−196°) in two different intestinal tapeworms, *Taenia hydatigena*[9] and *Moniezia expansa*[10]. Inorganic phosphate facilitates malate penetration into Ascaris muscle mitochondria by activating a carrier system[11] analogous to mammalian mitochondria[12–13]. The ability to carry out oxidative phosphorylation coupled with the existence of either a classical or modified mammalian-type respiratory chain system are two essential criteria favouring an aerobic metabolism. Moniezia was previously shown to have a modified electron transport chain with cytochrome *o* and cytochrome a_3[10] but oxidative phosphorylation was not demonstrated in this tapeworm or in any other intestinal parasite.

This paper reports some properties of Moniezia mitochondria with emphasis on mitochondrial structure, oxidative phosphorylation and effect of various respiratory inhibitors.

MATERIALS AND METHODS

Chemicals

Antimycin A (Type III), oligomycin and the sodium salts of ADP, ATP, NADH (from yeast), glutamate, malate, pyruvate and succinate were obtained from Sigma; sodium salts of L-ascorbate, EDTA and tetramethyl-*p*-phenylenediamine (TMPD) from British Drug Houses; atractyloside (potassium salt) from Calbiochem and other reagents were of analytical grade. Crystalline *Bacillus subtilis* proteinase (Nagarse) was obtained from Teikoku Chemical Co., Osaka, Japan.

Methods

Isolation of mitochondria

Adults of *M. expansa* were thoroughly rinsed in Ringers solution and used within 1–2 h after removal from the host. The tapeworms were minced with a pair of scissors for 5 min in isolation Medium A (pH 7.4) containing 100 mM KCl, 50 mM Tris–HCl, 1 mM ATP, 5 mM $MgCl_2$, 1 mM EDTA and dissolved Nagarse proteinase (2 mg/g wet wt. tissue). Homogenization (four strokes) was carried out with a Thomas teflon-pestle glass homogenizer with a clearance of 0.006–0.009 inch. The homogenate, diluted to 5.0 ml/g wet wt. tissue, was further homogenized (one stroke) before being centrifuged at 850 × *g* for 5 min at 2–4°. The supernatant was filtered through three layers of surgical gauze and the mitochondria were isolated by differential centrifugation. The 14000 × *g* pellet, resuspended in 5 ml Medium B (pH 7.4) containing 100 mM KCl, 50 mM Tris–HCl, 0.2 mM ATP, 1 mM $MgCl_2$, 0.2 mM EDTA and 0.5 % bovine serum albumin, was first centrifuged at 850 × *g* for 5 min and the supernatant from this was recentrifuged at 7000 × *g* for 10 min. The mitochondria-containing 7000 × *g* pellet was then washed three times with Medium B

(without bovine serum albumin) before use. This method was also employed for isolating mitochondria from the ox neck muscle previously stored for 144 h post-mortem at 4°.

Electron microscopy

Thin sections and unfixed negatively-stained preparations of Moniezia mitochondria were examined. The fixation and treatment of the specimens for thin sections were carried out as described by ALLMANN *et al.*[14] but without acrolein, and Epon 812[15] was used for embedding. The mitochondria were negatively stained with 2 % phosphotungstic acid (pH 6.8).

Enzymic analyses

Oxygen uptake was measured polarographically with a Clark oxygen electrode [Yellow Spring Biological Oxygen Monitor (Model 53)] at 25°. The reaction medium (pH 7.2) contained 1.0 mM EDTA, 30.0 mM KCl, 6.0 mM $MgCl_2$, 75.0 mM sucrose and 20.0 mM KH_2PO_4. The ADP/O ratio and the respiratory control index were calculated from the electrode traces as described by CHANCE AND WILLIAMS[16]. Protein was determined by Folin–phenol reagent[17].

RESULTS

Mitochondrial structure of Moniezia

Fig. 1 illustrates thin sections of mitochondria isolated from Moniezia (A) and the ox neck muscle (B) which had previously been subjected to 144 h storage at 4°. Moniezia mitochondria have clearly defined outer and inner membranes, outer compartment and intracristal spaces, all of which were also observed in the ox neck muscle mitochondria. The existence of a double-membrane structure in Moniezia mitochondria is very convincingly demonstrated in the unfixed negatively-stained sample (C). Analogous with the classical mammalian mitochondria, the cristae of Moniezia mitochondria (D) show attached elementary particles.

Mitochondrial respiratory activities

The oxidation of various substrates by intact Moniezia mitochondria was stimulated by ADP. Table I shows the State 3 respiratory rates of various substrates tested. NADH failed to stimulate oxygen uptake showing that intact Moniezia mitochondria are impermeable to NADH, a property also found with carefully prepared intact mammalian mitochondria[18].

Oxidative phosphorylation

Moniezia mitochondria oxidize succinate and α-glycerophosphate with an ADP/O ratio of about 1.5, indicating that there are probably two phosphorylation sites associated with the oxidation of each of these substrates. Fig. 2 illustrates a typical oxygen trace demonstrating the stimulation of succinate oxidation by ADP (Trace A). This classical State 3 to State 4 transition[16] could be repeated several times (not shown) giving an average ADP/O ratio value of 1.5 and a respiratory control index of 1.6. The respiratory control index is low compared with freshly prepared mammalian mitochondria. This value, however, corresponds very well with the value of 1.8

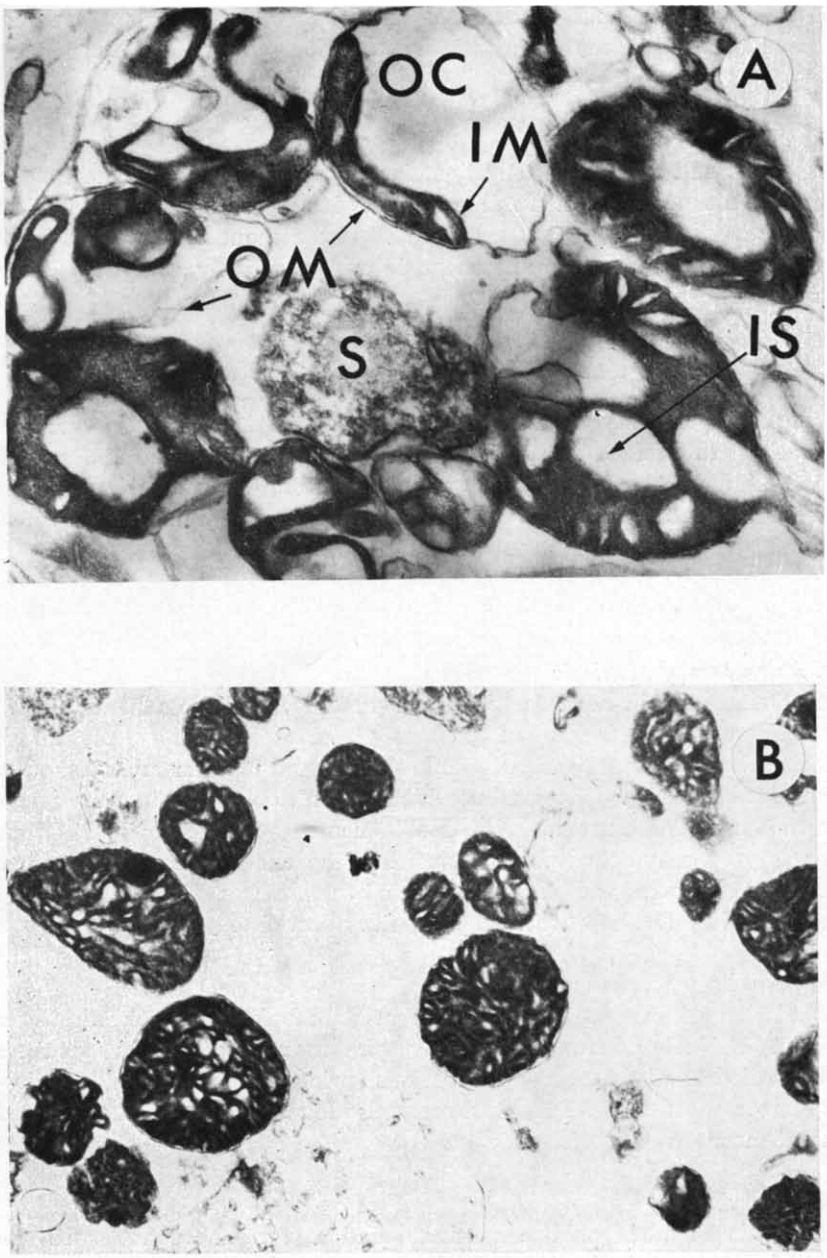
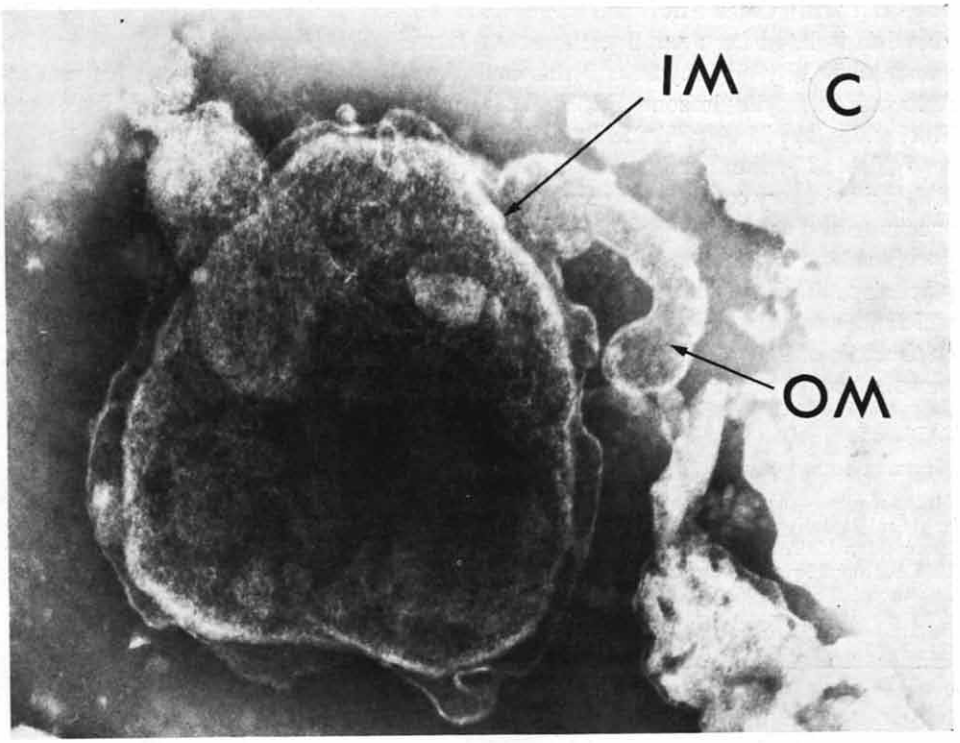


Fig. 1. Electron micrographs showing thin sections of Moniezia and ox neck muscle mitochondria and the unfixed negatively-stained Moniezia mitochondria. With the exception of a few swollen mitochondria all the intact mitochondria isolated from Moniezia (A) and the ox neck muscle (B) are in the condensed configuration. The unfixed negatively-stained samples of Moniezia mitochondria show clear definition of outer and inner membranes (C) and the existence of elementary particles (D). OM outer membrane; IM, inner membrane; OC, outer compartment; IS, intracristal space; S, swollen; EP, elementary particles. Magnification: A, 40000 ×; B, 10000 ×; C and D, 80000 ×. Electron microscopy was carried out in collaboration with Mr. C. A. Voyle.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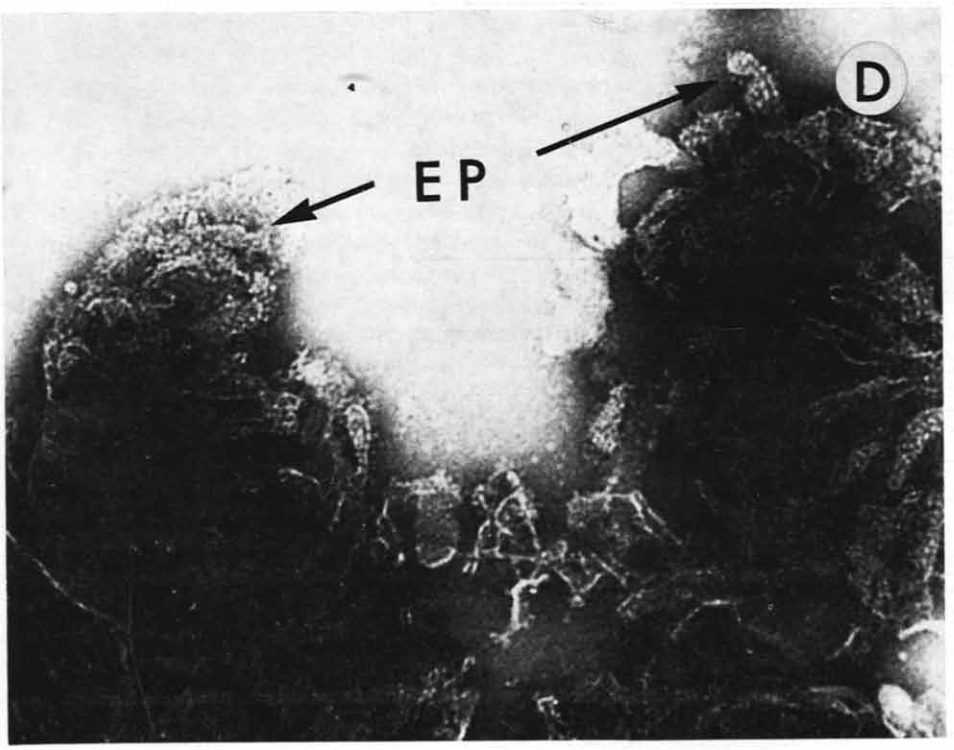
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observed with the ox neck muscle mitochondrial succinoxidase system for mitochondria isolated from 144 h post-mortem tissue[19]. The State 3 succinate respiration was inhibited by oligomycin and this could be relieved by *p*-trifluoromethoxycarbonylcyanidephenylhydrazone (FCCP) (Trace A). 50 % of the FCCP-uncoupled rate was blocked by 24 μM CN^- (Trace B).

Table II summarizes the data on oxidative phosphorylation, oligomycin sensitivity, respiratory control index and the FCCP-uncoupled rates of α-glycerophosphate, succinate and ascorbate–TMPD oxidation. With ascorbate–TMPD, no clear cut State 3 to State 4 transition was observed. Oxidative phosphorylation was definitely taking place since the State 3 rate of ascorbate–TMPD oxidation was inhibited by oligomycin which was subsequently uncoupled by FCCP. The respiratory control index, based on the ratio of the ADP-induced rate divided by the rate in the presence of oligomycin, was 1.4, as compared with about 2 for α-glycerophosphate and succinate.

TABLE I

MONIEZIA MITOCHONDRIAL RESPIRATORY ACTIVITIES

All the respiratory activities were measured polarographically with a Clark oxygen electrode at 25 in 2.5 ml (total volume). The data represent an average value from three separate State 3 rates induced by ADP. Reaction medium (mM): KCl, 30.0; $MgCl_2$, 6.0; sucrose, 75.0; KH_2PO_4, 20.0; EDTA, 1.0 (pH 7.20). Final concentration of substrates (mM): α-glycerophosphate, 8.0; succinate, 8.0; ascorbate, 4.0; TMPD, 0.2; L-malate, 8.0; pyruvate, 8.0; L-glutamate, 8.0; NADH 1.0. Rotenone (2 μM) was added prior to succinate in estimating the succinoxidase activity and 0.1 μg antimycin A per mg protein before TMPD addition for the ascorbate–TMPD oxidase activity.

Oxidase system	*State 3 rate (natoms O per min per mg protein)*
α-Glycerophosphate	78
Succinate	52
Ascorbate *plus* TMPD	52
L-Malate	22
Pyruvate *plus* L-malate	16
L-Glutamate *plus* L-malate	16
L-Glutamate	12
Pyruvate	6
NADH	0

TABLE II

OXIDATIVE PHOSPHORYLATION IN MONIEZIA MUSCLE MITOCHONDRIA

Experimental details as described in the legends to Table I and in MATERIALS AND METHODS except that the final concentration of oligomycin and FCCP was 1 μg per mg protein and 1 μM, respectively. The respiratory control index values, within parentheses, were calculated by dividing the State 3 rate by the oligomycin inhibited rate.

Oxidase system	*Oxygen uptake (natoms O per min per mg protein)*			*ADP/O*	*Respiratory control index*
	ADP	*ADP + oligomycin*	*ADP + oligomycin + FCCP*		
α-Glycerophosphate	78	42	90	1.4	1.4 (1.9)
Succinate	52	26	69	1.5	1.6 (2.0)
Ascorbate *plus* TMPD	52	32	66	–	– (1.4)

Effect of ADP on succinate oxidation

The effect of ADP concentration on succinate oxidation (State 3 rate) by Moniezia mitochondria is shown in Fig. 3. The amount of ADP required to give half-maximal acceleration of succinate oxidation (K_m) is about 28 μM, as compared with 26 μM ADP for the succinoxidase system of pigeon heart mitochondria[20] and 20–30 μM ADP found for liver mitochondria[20].

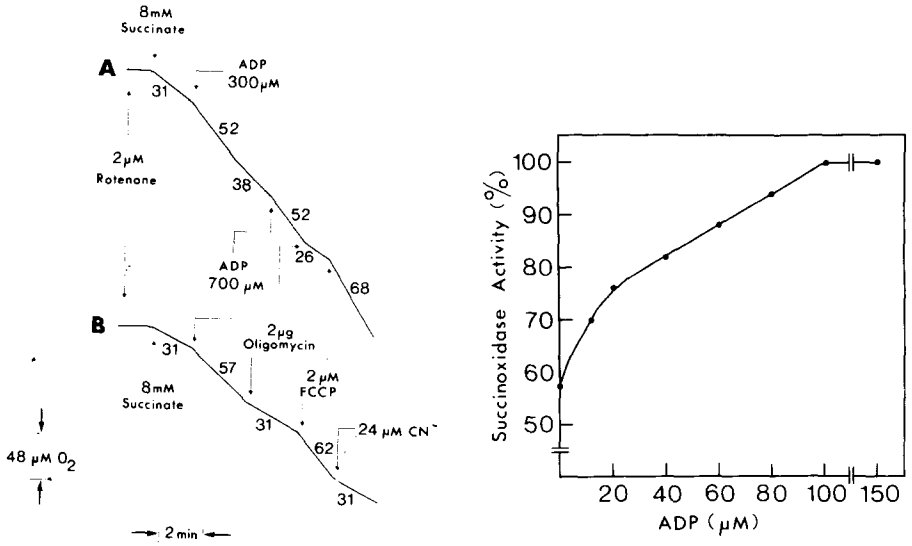


Fig. 2. Typical oxygen electrode tracings showing respiratory control (Trace A) and effect of oligomycin, FCCP and CN^- (Trace B) on Moniezia mitochondrial succinoxidase system. All respiratory rates, expressed in natoms O per min per mg protein, adjacent to the electrode traces, were estimated with a Clark oxygen electrode. Total protein, 3.07 mg. Other experimental details are given in Table I.

Fig. 3. Effect of ADP on Moniezia mitochondrial succinate oxidation. The succinoxidase activity (State 3) was determined polarographically at 25° as described in Table I and Fig. 2.

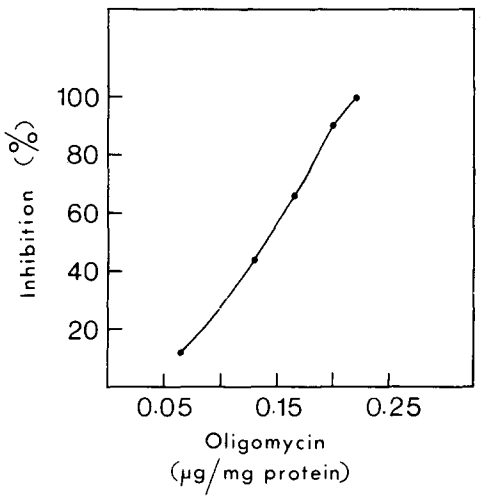
Effect of oligomycin and atractyloside on oxidative phosphorylation

Oligomycin is widely employed for inhibiting the synthesis of ATP by mitochondrial respiratory chain system without affecting substrate-linked phosphorylation[21] and atractyloside as a specific inhibitor of adenine nucleotide translocator for the formation of exogenous mitochondrial ATP[22]. The State 3 rate of α-glycerophosphate, succinate and ascorbate–TMPD oxidation was inhibited by oligomycin. The sensitivity of succinate oxidation to this inhibitor is shown in Fig. 4, where complete block of the ADP-stimulated rate was observed at a concentration of about 0.23 μg oligomycin per mg mitochondrial protein, as compared with 0.18 μg oligomycin per mg rat liver mitochondrial protein[23]. 50 % inhibition of oxidative phosphorylation with succinate as substrate was achieved at 0.14 μg oligomycin per mg Moniezia mitochondrial protein, and 92 % inhibition with 0.5 μg atractyloside per mg protein in the presence of 1.2 mM ADP.

Effect of piericidin A on NAD^+-linked oxidation

The oxidation of pyruvate *plus* malate was used to study the inhibitory affect

of piericidin A on Moniezia mitochondrial NAD^+-linked oxidation. A complete block of the ADP-stimulated rate of pyruvate *plus* malate oxidation required about 50 pmoles piericidin A per mg protein (Fig. 5), an amount similar to that observed for the complete inhibition for the State 3 rate of pyruvate *plus* malate oxidation in mitochondria from the back muscle of the Pietrain pig [4].



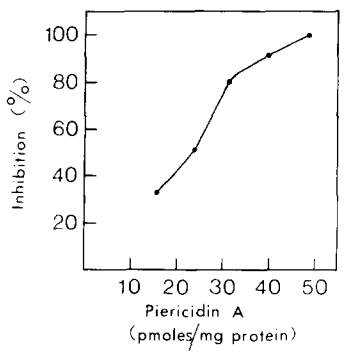


Fig. 4. Inhibition of oxidative phosphorylation in Moniezia mitochondria by oligomycin. The effect of oligomycin on Moniezia oxidative phosphorylation was tested using succinate as substrate. All respiratory activities were estimated polarographically at 25° as described in Table I and Fig. 2. % inhibition by oligomycin was calculated on the ADP-stimulated rate (State 3) since oligomycin only inhibits that part of respiration coupled to phosphorylation.

Fig. 5. Inhibition by piericidin A on the State 3 respiratory rate of pyruvate *plus* malate oxidation by Moniezia mitochondria. Piericidin A was added prior to ADP (500 μM) to block the State 3 rate of pyruvate (8 mM) *plus* malate (8 mM) oxidation. Other details as described in Table I and Fig. 2. % inhibition by piericidin A was calculated from the ADP-stimulated rate after correction was made for the rate of respiration in the absence of ADP.

Effect of antimycin A

Fig. 6 illustrates the sensitivity of the State 3 rate of the succinoxidase system towards antimycin A. 50 % inhibition was achieved at a concentration of 8 ng antimycin A (approx. 16 pmoles) per mg protein, and 67 % at 20 ng antimycin A (approx. 40 pmoles). Further increase in the concentration of this inhibitor did not produce a further increase in inhibition. The data suggest the existence of an antimycin A-insensitive pathway accounting for 33 % of the oxygen uptake in Moniezia mitochondria. With rat liver mitochondria, 68 % of the succinoxidase system was blocked by 33 ng antimycin A (66 pmoles) per mg protein and 100 % inhibition at 55 ng antimycin A (110 pmoles) per mg protein[25].

Effect of CN^-

The sensitivity of Moniezia mitochondrial respiratory chain system towards CN^- was tested using three different electron donors (Figs. 7A–7C). The State 3 rate of succinoxidase activity was inhibited 84 % by 42 μM CN^-; further increase of CN^- concentration up to 90 μM did not inhibit further. CN^- was found to be less effective

in the presence of FCCP. Thus, 42 μM CN^- only blocked 63 % of the FCCP-uncoupled succinoxidase activity instead of 84 % in the absence of FCCP. The K_i, *i.e.* the amount of CN^- required to give a 50 % inhibition of the succinoxidase system was increased from 8 μM (without FCCP) to 24 μM (with FCCP). The same type of phenomenon, which appears to be a general property of uncouplers[26], was also observed

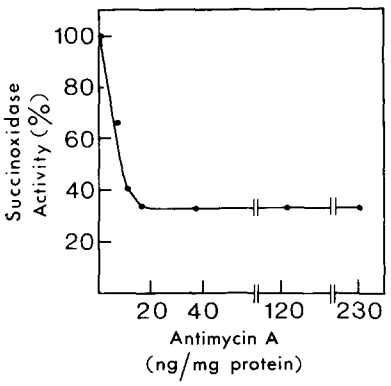


Fig. 6. Effect of antimycin A on the succinoxidase activity (State 3) of Moniezia mitochondria. The inhibitor in 95 % ethanol was added after ADP (600 μM). The control experiment contained the same volume of ethanol but without antimycin A. Other details as described in Table I and Fig. 2.

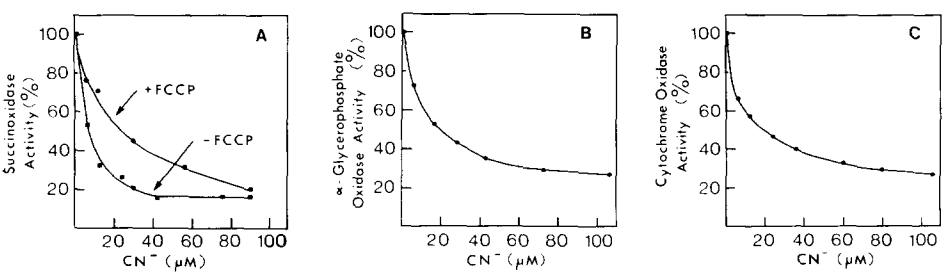


Fig. 7. Effect of CN^- on succinoxidase, α-glycerophosphate oxidase and cytochrome oxidase activities of Moniezia mitochondria. The % inhibition of the succinoxidase (A), α-glycerophosphate oxidase (B) and cytochrome oxidase (C) activities were determined polarographically at 25° in a total volume of 2.5 ml. Other experimental details are given in Table I and Fig. 2.

with rat liver mitochondria oxidizing succinate in the presence of FCCP but N_3^- was employed[26] instead of CN^-. Both the α-glycerophosphate (B) and cytochrome oxidase (C) activities (State 3) were also CN^- sensitive. The K_i for both these systems, in the presence of FCCP, was about 20 μM CN^-, a value almost identical to that of the succinoxidase system. Furthermore, the same extent of inhibition (63 %) was obtained for both the α-glycerophosphate and cytochrome oxidase activities with 42 μM CN^- in the presence of FCCP.

DISCUSSION

Carefully prepared Moniezia mitochondria have similar structural characteristics to aerobic mitochondria, and are also capable of carrying out oxidative phosphoryla-

tion with the ADP/O ratio values comparable to the classical mammalian system for succinate and α-glycerophosphate oxidation. Oligomycin and atractyloside also effectively inhibited oxidative phosphorylation. These data suggest that oxygen is essential for energy production in Moniezia mitochondria. The classical respiratory chain inhibitors (piericidin A, antimycin A and CN^-) were found to be equally inhibitory with Moniezia mitochondria as with strictly aerobic mammalian mitochondria. Only antimycin A showed a slight variation in that 33 % of the Moniezia respiratory chain system was antimycin A insensitive. A concentration of 230 ng antimycin A (460 pmoles) per mg mitochondrial protein produced the same extent of inhibition as 20 ng antimycin A per mg protein, whereas with strictly aerobic mammalian mitochondria, almost complete inhibition was attained at 230 ng antimycin A per mg protein[25].

The success in demonstrating oxidative phosphorylation in Moniezia coupled with previous data[10] on the existence of functional cytochromes of type *b* (including cytochrome *o*), *c* and *a* (including cytochrome a_3) further substantiated the hypothesis[5] that large intestinal parasites are really aerobic. This viewpoint is further strengthened by the recent success in showing the existence of functional cytochrome a_3 in *T. hydatigena*[9] and Ascaris[8]. Furthermore, cytochrome oxidase activity, determined polarographically with ascorbate *plus* TMPD as electron donors to the *c*-type cytochrome of the respiratory chain system[27], was also detected in another tapeworm, *Hymenolepis diminuta*. This observation supports that previously reported by READ[28] whose finding was questioned and contradicted by SCHEIBEL *et al.*[3] who were unable to detect any cytochrome oxidase activity in *H. diminuta*.

The accumulated data on various intestinal parasites[8–10] obtained by using highly sensitive spectrophotometric techniques favour large intestinal parasites to be aerobic. This does not necessarily imply that they must have the same aerobic metabolic patterns as the classical mammalian tissue. On the contrary, one would tend to expect modifications of the classical mammalian respiratory pattern for adaptation to the low oxygen environment. Their respiratory chain systems would probably be modified by having more than one terminal oxidase. The evidence[8–10] so far supports the participation of cytochrome *o* and cytochrome a_3 in functioning as terminal oxidases in large intestinal parasites.

The discrepancy regarding the existence of functional cytochromes in large intestinal parasites in earlier published findings[1, 3–4, 29–30] is mainly due to inadequate or even incomplete experimentation. A good example of the latter is the earlier misleading postulation that a functional *c*-type cytochrome might not be present in Moniezia[31]. By more careful experimentation using spectrophotometric techniques at liquid N_2 temperature (−196°), cytochrome *c* and cytochrome c_1 were subsequently shown to be present and also functional in Moniezia[10]. Furthermore, cytochrome *c* was recently highly purified (88 % pure) from this particular tapeworm (unpublished data) and at liquid N_2 temperature, the α-band of the reduced pigment (absolute spectrum) showed two satellite peaks at 545.5 and 534 nm. The β-band split into four satellite peaks at 524, 517.5, 511 and 507 nm. Purified Ascaris cytochrome *c*, on the other hand, showed satellite absorption peaks (−196°) at 548 and 542 nm (α-band) and 528, 521 and 511 nm (β-band). When compared with cytochrome *c* from mammalian tissue purified to the same extent, the α-band (−196°) showed three satellite peaks at 547 (c_{α_1}), 544.5 (c_{α_2}) and 537 (c_{α_3}) nm (unpublished data). Thus, Moniezia

cytochrome *c* appears not to have the $c_{\alpha 1}$ peak (—196°) and with Ascaris cytochrome *c*, the $c_{\alpha 3}$ peak might not be present.

The possibility that all these observations particularly with regard to oxidative phosphorylation could be artefacts is ruled out by our recent data obtained for mammalian skeletal muscle. Mitochondria in the ox neck muscle, after having been subjected to anaerobiosis for about 144 h *in situ*, could still be isolated intact, and also retained their capacity for oxidative phosphorylation[19].

ACKNOWLEDGEMENT

The author wishes to express his thanks to A. M. Cheah for technical assistance, to Dr. P. B. Garland for the gift of piericidin A and Dr. P. Heytler for FCCP.

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