Isolation and Respiratory Assay of Earthworm Body Wall Mitochondria

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Out of several media tested, KCl medium containing BSA was found to be most suitable for isolation of functionally efficient mitochondria from earthworms. Respiration and oxidative phosphorylation in isolated mitochondria are demonstrated in the presence of cytochrome *c* and BSA in reaction medium.

Introduction – The details of the respiratory metabolism in earthworm are yet to be established. There are only a few contradictory reports on the nature of its respiratory enzyme components [1]. While, cytochrome oxidase, succinoxidase, succinate dehydrogenase were identified in earthworm tissue homogenates, the presence of Kreb's cycle enzymes could not be demonstrated. Very little information is available on earthworm mitochondria except one report where it is isolated in 0.44 M mannitol [2]. Mitochondria from invertebrates like insects and crustaceans are isolated and characterized [3, 4]. But such methods cannot be extended to earthworms as it is known that conditions for isolation and assay of mitochondria are highly system specific [5].

In view of this, the present report compares different isolation and reaction media for mitochondrial assay in earthworms.

Materials and Methods

Tissue preparation and isolation of mitochondria

About 15–20 laboratory reared earthworms *(Octochaetona surensis)* immobilized on crushed ice were dissected to free the body wall from alimentary canal and other tissues. After rinsing clean in ice cold isolation medium, the muscles were gently pound into a paste in cold mortar and pestle, and then homogenised in Potter Elvehjem homogenizer at 2000 rpm for 30 s (6 strokes). All these operations were carried out at $4 \pm 1°$. The homogenate was centrifuged at $600 \times g$ for 5 min to remove the debris. The homogenate supernatant was cenrifuged at $8000 \times g$ for 20 min in a refrigerated centrifuge (model K-24, Remi Udyog Ltd, India). Mitochondrial pellet was rinsed with little amount of isolation medium and resedimented at $8000 \times g$ for 10 min to remove contaminations. The washing procedure was repeated twice before suspending the mitochondrial pellet in isolation medium, by a loose fitting Potter-Elvehjem homogenizer. Protein was measured by Lowry's [6] method using BSA as standard. Isolation media were (i) 0.25 M sucrose (ii) 0.44 M mannitol (iii) 0.44 M mannitol, 0.02 M Tris-HCl buffer pH 7.4 and 0.001 M EDTA (iv) 0.13 M KCl, 0.02 M Tris-HCl buffer pH 7.4, 0.001 M EDTA (v) 0.13 M KCl, 0.02 M Tris-HCl buffer pH 7.4, 0.001 M EDTA, 0.5% BSA and 0.001 M Na_2 ATP.

Measurement of oxygen uptake

The mitochondria (3–5 mg protein/ml) were used for assay immediately after isolation. Oxidative phosphorylation was measured at 30° using a polarograph with a clark type oxygen electrode (Universal Biochemicals Madurai, India). Reaction media for the assay were (i) 0.44 M mannitol, 10 mM potassium phosphate buffer pH 7.4, 20 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl buffer pH 7.4, when mannitol was used as extraction medium (ii) 0.15 M KCl, 10 mM Tris HCl buffer pH 7.4, 1 mM EDTA, 20 mM potassium phosphate buffer and 5 mM $MgCl_2$ when KCl isolation medium was used. Cytochrome C (0.01–0.5 mM) and BSA (0.5%) were added exogenously. Succinate (3.3 mM) was the substrate and mitochondrial suspension was added to initiate the reaction. ADP:O and respiratory control index were calculated according to Estabrook [7]. Rat liver mitochondria were isolated in 0.25 M sucrose and were assayed at 30°.

Results and Discussion

Earthworm mitochondria isolated in non-electrolyte media are functionally not very efficient. Only those isolated in KCl medium show the normal characteristics of mitochondria (Table I, Fig. 1). In this respect, they resemble the insects [8] except that exogenous cytochrome *c* has to be supplied in earthworm systems. Exogenous cytochrome *c* has been known to stimulate respiration in insects and rat skeletal muscle mitochondria [3, 5]. But, while it increases the oxygen uptake by 80% in insect, in earthworm mitochondria there is no

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Table I. Oxidative phosphorylation in earthworm body wall mitochondria.

Isolation medium[a]	Reaction medium[b]	O_2 consumption			
		nmol/mg protein/min		RCI	ADP/o
		State IV	State III		
mannitol	mannitol +0.1 mM cyt. *c*	21.57	[c]	[c]	[c]
mannitol Tris & EDTA	i) mannitol +0.1 mM cyt. *c*	38.52	[c]	[c]	[c]
	ii) mannitol +0.1 mM cyt. *c* +0.5% B.S.A.	39.74	[c]	[c]	[c]
KCl, Tris & EDTA	KCl +0.01 mM cyt. *c*	39.92	[c]	[c]	[c]
KCl, Tris, EDTA, 0.5% B.S.A. & 1 mM Na-ATP	KCl +0.01 mM cyt. *c* +0.5% B.S.A.	22.05	40.41	1.22	1.85
Rat liver mitochondria					
Sucrose	Sucrose	4.44	26.6	1.38	5.9

[a, b] Isolation and reaction media as described in the text; [c] could not be calculated.

respiration in its absence (Table I). Probably due to its location and shuttling nature most of the cytochrome *c* in earthworm mitochondria is leaked out during isolation. Alternatively, it might be inactivated during isolation and hence ineffective in electron transport. However it is clear that the isolation medium influences the cytochrome *c* requirement in earthworm mitochondria (Table I). Possibly the ionic medium increases the rate of reconstitution of cytochrome *c* in inner membrane and it requires less amount of it, than the mannitol isolation medium.

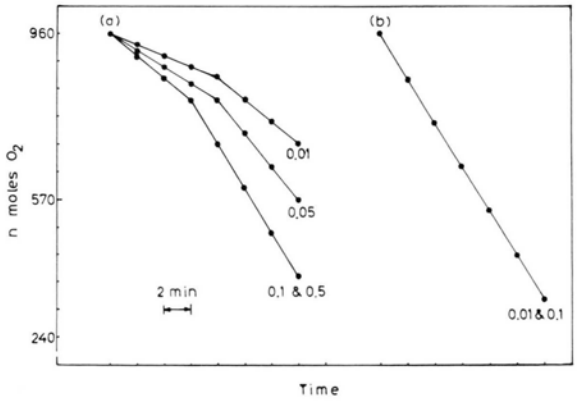

Fig. 1. Oxygen uptake by earthworm mitochondria isolated in (a) Mannitol-Iris-EDTA (b) KCl-Tris-EDTA. Reaction media as described in the text, contain 3.3 M succinate and mitochondrial equivalent of 4.6 mg protein. Cytochrome *c* (in mM) addition are indicated above the traces.

The ability of BSA to bind with various toxic substances produced during membrane damage and consequently stabilising the membrane structure, has been reported earlier [9]. Also it has been reported that Na_2ATP in isolation medium stimulates state 3 respiration in rat skeletal muscle mitochondria [5]. Similar to these findings in earthworm mitochondria, BSA and Na_2ATP can restore respiratory control in KCl extracted medium, but not in mannitol. This differential response can be attributed to the extent of membrane damage in different isolation media.

It is difficult to demonstrate respiratory control in several invertebrate systems during *in vitro* assay of mitochondria [4, 10]. In insects, the absence of respiratory control is due to the limited permeability of Krebs cycle intermediates into mitochondria and also it accounts for its low ADP:0 ratio of 0.4 with succinate [11]. In earthworm mitochondria, the ADP:0 ratio of 1.22 and respiratory control index of 1.85, evidences its efficiency in utilising the Krebs cycle intermediates.

From these observations it is clear that active mitochondria can be isolated from earthworm body wall muscle in KCl medium containing BSA. Oxidative phosphorylation in these mitochondria can be assayed in a reaction mixture having BSA and cytochrome *c* in addition to other basic components.

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