OXIDATIVE PHOSPHORYLATION BY ISOLATED MITOCHONDRIA OF *NEUROSPORA CRASSA*

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To our knowledge oxidative phosphorylation has not been reported to occur in particulate fractions isolated from any fungi other than yeasts which, until now, have been the main organism used in studying mitochondrial biogenesis (see Linnane, 1964, for references). Thus in order to initiate a correlated investigation into the structural and biochemical development of mitochondria in *N. crassa*, which has certain cultural and genetic advantages, we have developed a procedure to isolate functional mitochondria. The criterion used to identify the structures isolated in our study as being mitochondria was the ability to couple phosphorylation to oxidative activities. The procedure which we have developed enables us to isolate large quantities of relatively pure mitochondria from conidial, germinating and stationary phases of growth of the organism; this greatly facilitates the biochemical, chemical and structural studies presently being undertaken.

The present paper clearly establishes that mitochondria of *N. crassa* isolated by our procedure carry out oxidative phosphorylation. Preliminary observations showing biochemical differences in mitochondria isolated at different stages of growth of the organism are also presented.

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Methods - - - Conidia of N. crassa, wild type strain SY7A, grown and harvested essentially as described by Wainwright (1959) were stored up to one week in distilled water at 4°C without loss of viability. Approximately 10^{10} conidia were added to each 2 liter Erlenmeyer flask containing 500 mls of Vogel's complete growth medium (Vogel, 1956) plus 3 drops of silicone antifoam and were incubated on a rotary shaker at 30°C. To obtain hyphae at 3 1/2 hours, sucrose was omitted from the growth medium; in the presence of sucrose conidia have not germinated at 3 1/2 hours, whereas in the absence of sucrose hyphae have an average length of 9μ at this time. All subsequent operations were performed at 0-4°C.

The conidia or hyphae were collected by centrifugation at 500 x g for 5 minutes and resuspended in a preparation medium consisting of sucrose, 0.25 M; EDTA, 0.005 M; and crystalline bovine serum albumin (BSA), 0.15%. A 250 ml volume of conidia or hyphae in preparation medium plus 500 g of glass beads (0.2 mm) and 4 drops of silicone antifoam were added to a Gifford-Wood Eppenbach Micro-Mill (Model MV-6-3) and ground at a maximum speed for 1 minute (gap setting = 30 thousandths of an inch). The ratio of liquid to bead volume and grinding times are critical to obtain satisfactory preparations; under the conditions described here about 75% breakage of hyphae is obtained. The mitochondria were collected by differential centrifugation between 1,500 and 8,000 x g. A second 1,500 x g centrifugation before finally washing and collecting the mitochondria at 8,000 x g removes excessive cell wall material. All fractions were negatively stained with potassium phosphotungstate, pH 6.8, and surveyed in the electron microscope in order to rule out the possibility that the mitochondria were grossly contaminated with other subcellular components.

The mitochondrial pellet was resuspended in the preparation medium to give a final concentration of 20-30 mg protein/ml. Protein was determined by the microbiuret method (Goa, 1953) using albumin as a standard. Oxidative phosphorylation was determined by measuring respiration manometrically at 25°C (Slater and Holton, 1954) and by determining the inorganic phosphate

uptake.

Results - - - The data in Table I show the capacities of mitochondria isolated from organisms which were harvested at 3 different stages of development to perform oxidative phosphorylation using 7 different respiratory substrates. Optimum P/O ratios were obtained at pH 6.5 and 6.9. The requirement for the individual components of the reaction mixture in the oxidation of succinate was shown in other experiments.

Table I

Oxidative Phosphorylation by Isolated Mitochondria

The reaction mixture contained 125 mM sucrose, 10 mM $MgCl_2$, 10 mM Na-phosphate pH 6.9, 1.5 mM EDTA, 1 mM ADP, 20 mM glucose, 300 K.M. units hexokinase, 6.3 mg BSA and one of the following substrates: 25 mM ascorbate + 0.3 mM TMPD, 10 mM each succinate, citrate, isocitrate, α-ketoglutarate, pyruvate + 2.5 mM malate (all plus 1 mM DPN, except ascorbate), or 10 mM DPNH. 3-7 mg mitochondrial protein was added in a total volume of 2 ml. Incubation was at 25°C. Uptake data in μmoles P_i or μatoms O_2/mg protein/hour. The zero values for oxidation of 4 substrates with conidial mitochondria are relative since no O_2 uptake was detected manometrically; however, polarographically very low rates of O_2 uptake were detected with these substrates. All figures are rounded off.

		CONIDIA	3 1/2 HR. HYPHAE	2 DAY HYPHAE
SUBSTRATE				
Ascorbate	P_i	2.7	5.6	5.2
	O_2	7.6	11.6	8.4
	P/O	0.4	0.5	0.6
DPNH	P_i	3.6	5.1	5.8
	O_2	6.4	13.9	6.9
	P/O	0.6	0.4	0.8
Succinate	P_i	0.5	3.8	7.5
	O_2	0.7	2.7	5.0
	P/O	0.7	1.4	1.5
Pyruvate (Malate)	P_i	0	0.6	0.8
	O_2	0	0.8	0.4
	P/O	0	0.8	1.9
Citrate	P_i	0	2.1	7.6
	O_2	0	2.3	2.6
	P/O	0	0.9	3.0
Isocitrate	P_i	0	0.4	2.4
	O_2	0	0.4	1.5
	P/O	0	1.0	1.6
α-Keto-glutarate	P_i	0	1.8	4.7
	O_2	0	1.0	1.4
	P/O	0	1.7	3.3

The following compounds uncoupled phosphorylation from the oxidation of succinate by mitochondria isolated from two day old hyphae: dinitrophenol, 0.4 mM; oleate, 0.1 mM; gramicidin, 0.006 mM; carbonyl cyanide m-chlorophenylhydrazone, 0.0025 mM; carbonyl cyanide-p-trifluoro-methoxyphenylhydrazone, 0.001 mM; atractylate, 0.1 mM; and oligomycin, 2 μg/mg protein. Inhibition of oxidation of succinate was accomplished by KCN, 1 mM; antimycin A, 0.2 μg/mg protein; and malonate, 20 mM. The action of these inhibitors is similar to that obtained with mitochondria isolated from other organisms.

Mitochondria isolated at all three stages of development also showed the following biochemical activities: Mg^{++}-activated ATPase, ATP-ADP exchange, ATP-P_i exchange, Ca^{++} uptake, incorporation of leucine-C^{14} into protein, and respiratory control as measured polarographically; in addition, they contained cytochromes b, c and a.

Electron micrographic studies showed heterogeneity in the size and structural organization of individual mitochondria but no major differences have been observed when the mitochondrial fractions from the 3 stages of development are compared in thin sections or negatively stained preparations. A detailed cytological and ultrastructural comparison of Neurospora cells and isolated mitochondria (at different stages of growth of the organism) is in progress.

Discussion - - - By the inclusion of BSA in the isolation medium we have been able to isolate a mitochondrial fraction from Neurospora capable of performing oxidative phosphorylation. The omission of BSA and the inclusion of phosphate (which is known to be a mitochondrial swelling agent) may be the reason why other investigators have not been able to isolate mitochondria capable of coupling phosphorylation to oxidation. BSA was shown by Wojtczak and Wojtczak (1960) to be beneficial in the isolation of functional mitochondria from a number of organisms. They showed that the BSA removes fatty acids which uncouple oxidative phosphorylation and it is possible that BSA performs the same function in our Neurospora preparations.

The interesting biochemical differences between mitochondria isolated at the 3 different stages of growth could result from a number of factors, e.g., inherent differences in the mitochondrial structure and enzyme complement, differential loss of proteins during isolation, or, perhaps, a more active DPNase at the younger growth stages in the development of Neurospora mitochondria. However, the possibility that these differences reflect important biochemical stages in the development of Neurospora mitochondria must be considered.

Studies of mitochondrial development in yeast (Schatz, et al., 1963; Mahler, et al., 1964; Linnane, 1964) and Neurospora (Luck, 1963) have stimulated anew, various theories concerning the origin of mitochondria. We intend to evaluate these in the light of our present and future findings and hope to exploit the advantages of Neurospora in studying the developmental sequence of mitochondria and the possible role of structural-functional precursors in this development.

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