RESPIRATORY CONTROL IN MITOCHONDRIA FROM CRITHIDIA FASCICULATA

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Received November 15, 1971

SUMMARY. The trypanosomatid Crithidia fasciculata contains a single bifurcated mitochondrial organelle, and a method is described for its isolation as a relatively intact structure. These preparations carry out oxidative phosphorylation and show respiratory control with succinate as substrate. The oxidation and phosphorylation efficiency of succinate and α-glycerophosphate, as well as NAD^+-linked substrates, are affected by antimycin A, mCl-CCP, and rutamycin.

Ultrastructural studies of the trypanosomes which are sensitive to cyanide have shown the presence of a unique single mitochondrial organelle. This mitochondrion is remarkable with regard to its large size in relation to the cell and its physical association with the kinetoplast. The latter is a DNA-containing structure unique to the Trypanosomatodiae and Bodiniae and possibly implicated in mitochondrial biogenesis (1).

Crithidia fasciculata has been shown to be similar to other trypanosomatids in ultrastructure (2, 3) and to contain a bifurcated mitochondrial organelle whose extended branched length is at least two to four times that of the cell itself. The cytoplasm is surrounded by a pellicular membrane that is not easily ruptured. Therefore extraction of the mitochondrion as an intact structure has been an obstacle to in vitro investigation of its energy linked functions.

We have demonstrated oxidative phosphorylation in cell free homogenates of Crithidia (4), and a subsequent report has shown this capacity to reside, as expected, in a mitochondrial fraction (5). However, no mitochondria isolated from Crithidia have demonstrated respiratory control (RC), an index used for

"tightly coupled" mitochondria. In mammalian systems, it is known that the intactness of the mitochondrion is essential for the demonstration of RC and of a high efficiency of energy coupling. In the present report a method is presented for the isolation of a mitochondrial fraction from Crithidia showing RC.

EXPERIMENTAL METHODS

Crithidia fasciculata (A. T. C. C. No. 11745) was grown in liquid culture as previously described (6) except for the elimination of the boiling step and the addition of yeast extract (final conc. 0.1%) which was autoclaved separately as a 20% solution. The organisms were grown to a density of 1.5×10^8 cells/ml, rapidly chilled in ice, and harvested by centrifuging for 8 minutes at 750 x g. Cells were washed twice by resuspending in a MOPS-buffered isolation medium (MIM)* (approx. 13 ml MIM/gm wet weight) and centrifuging 8 minutes at 1600 x g. The cells were transferred to a chilled mortar. Chilled silicon carbide abrasive grain (Crystalon: Norton Company: grit No. 400B) was added to the mortar (approx. 60:40; gm Crystalon/gm wet weight) and the cells were ground until microscopic examination showed that approximately 80% of the cells were lysed. The suspension was then diluted with MIM and a mitochondrial fraction isolated according to Fig. 1. Overlay washes at (B) and (C) were performed by gently agitating the surface layer of the pellet by dropwise addition of MIM. Phase contrast and electron microscopy showed that the final mitochondrial pellet contained large mitochondrial structures and partially fragmented mitochondria, some kinetoplasts usually attached to mitochondrial arms, flagellar pieces, and a small amount of cellular debris.

Manometric assays and phosphate determinations were performed as previously described (4). Polarographic assays were carried out with a Clark-type

*MIM contained 0.35 M sucrose, 0.0025 M magnesium acetate, 0.001 M EGTA (ethylene glycol-bis(β-amino-ethyl ether) N, N'-tetraacetic acid), 0.15% bovine serum albumin (Fraction V), 0.02 M MOPS (morpholinopropane sulfonic acid) and was adjusted to a final pH of 7.7.

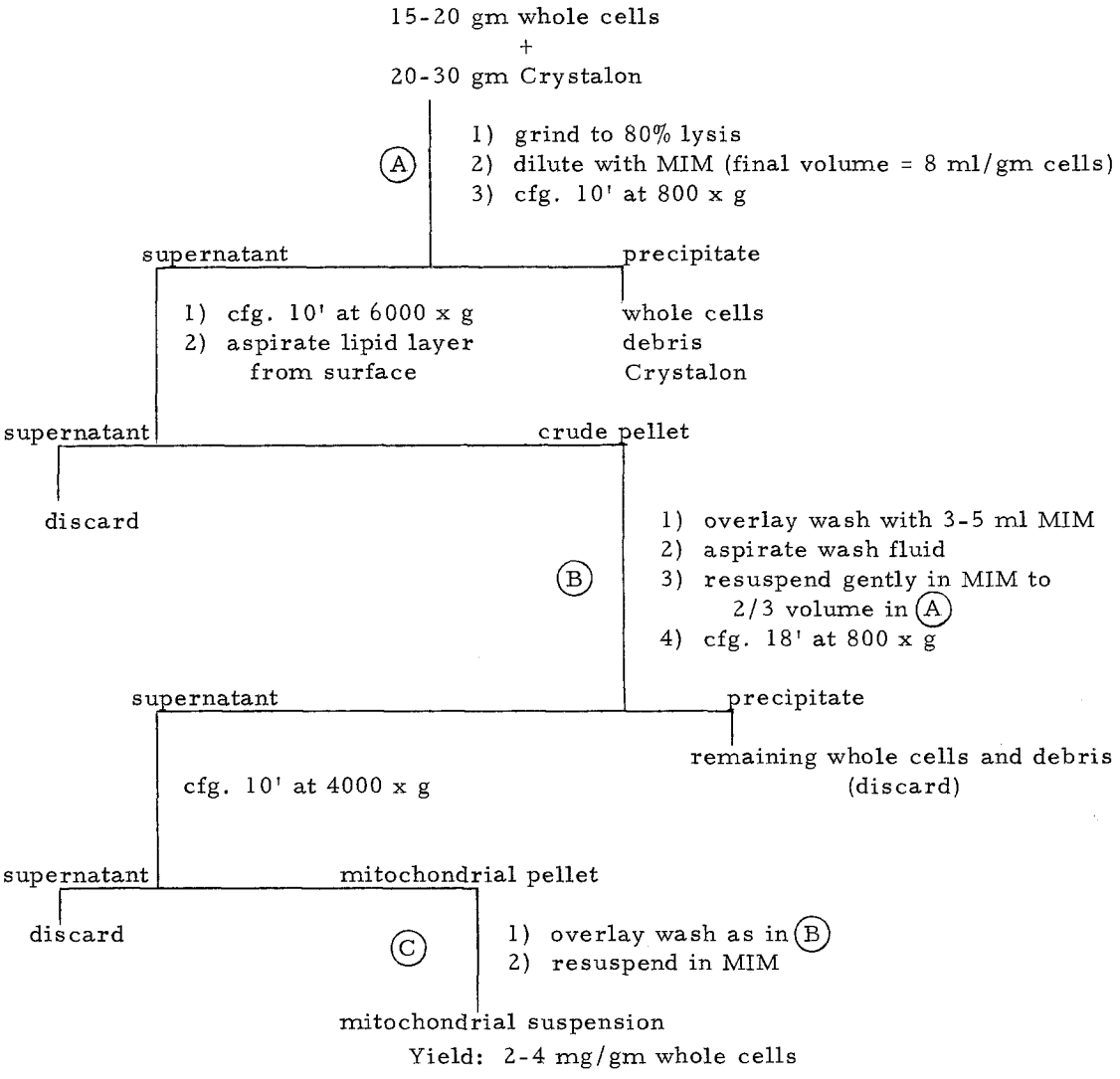

Fig. 1. Mitochondrial Isolation. All centrifugations performed in a Sorvall HB-4 rotor at 4°.

oxygen electrode (YSI Model 53: Yellow Springs Inst. Co.). The electrode was calibrated by the NADH-aliquot method described by Estabrook (7) and Chappell (8) except that Mycobacterium phlei electron transport particles (9) were used to measure NADH oxidation. Respiratory states and ADP:O ratios are defined and calculated according to Chance and Williams (10). Protein was determined

by the method of Lowry et al. (11). Sonic oscillation was performed with a Biosonik II (Bronwill) using the needle probe (No. BP-II-40T) at an intensity of 70. Most reagents were obtained from Sigma Chemical Co. Carbonyl cyanide m-chlorophenylhydrazone (mCl-CCP) (Calbiochem.) and rutamycin (a gift from Dr. R. Cockrell) were dissolved in dimethyl formamide (DMF). Antimycin A was dissolved in 95% ETOH.

RESULTS AND DISCUSSION

One of the criteria used in assessing the intactness of the isolated mitochondria was differential inhibition of succinoxidase by oxalacetate (OAA) and malonate. Although both compounds are competitive inhibitors of the particulate and soluble succinate dehydrogenase, in intact phosphorylating mammalian mitochondria the succinate dehydrogenase compartment is inaccessible to exogenous OAA whereas malonate permeates easily (12, 13, 14). As seen in Fig. 2, trace A, mitochondria as isolated from Crithidia showed a similar differential effect. However, as seen in trace B, after sonication succinoxidase activity was inhibited by OAA, and there was a loss of stimulation by ADP.

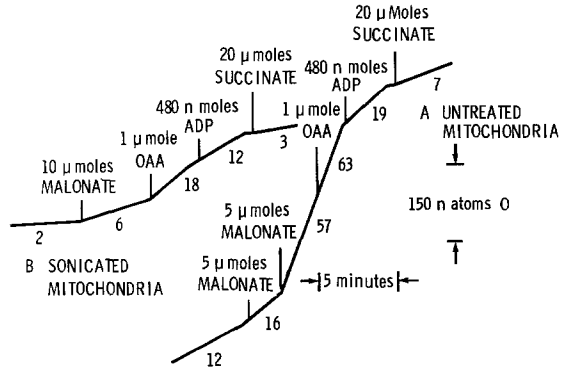

Fig. 2. Effect of oxalacetate and malonate on succinoxidase activity. Reaction vessels contained (final concentration) 0.32 M sucrose, 0.11 M MOPS, pH 7, 5 mM KPO_4, 0.9 mM EGTA, 2.2 mM Mg^{++} acetate, 1.3 mg of BSA, and 1.45 mg of mitochondrial protein in a total volume of 3.1 ml. The reactions were carried out at 20°. Reaction A contained untreated mitochondria and B contained mitochondria exposed to sonic oscillation for 60 sec. Respiratory activities adjacent to the electrode traces are expressed as natoms of oxygen per min. per mg protein.

As seen in Fig. 3, trace A, these mitochondria exhibit RC and were sensitive to rutamycin and mCl-CCP.

In the course of developing the isolation and assay conditions, it became apparent that the osmolarity and the specific ionic environment were important factors in the demonstration of RC. A sucrose concentration of at least 0.35 M and a Mg^{++} concentration of 2.5 to 3 mM were found to stabilize the mitochondrial vesicles during the grinding and centrifugation procedures.

In studies performed at 30°, certain anions including Cl^-, $SO_4^=$, and F^- were found to be inhibitory to the degree of ADP-stimulation of succinoxidase activity, i.e., State 4 $\longrightarrow$ 3 transition. Acetate was less inhibitory than the above and thus was selected as the Mg^{++} salt. The anionic inhibition also suggested the use of a zwitterionic substituted amino acid buffer which could be adjusted to the desired pH with base. Several "Good" buffers (15) including MOPS, TES, HEPES, PIPES, MES, BES, and TAPS were tested in reaction mixtures. Those (with suitable pKa's) exhibited little difference in State 3:4 ratios near a neutral pH optimum, but differed considerably in the more alkaline range of pH 7.5 to 8. The use of either KOH or NaOH as the neutralizing base was found to have no discernible difference on RC or P:O ratios.

Assay temperature was of importance in the demonstration of RC. As can be seen by comparison of the data in Fig. 3, RC ratios at 30° were decreased considerably over those at lower temperatures. There was a disproportionate increase in State 4 respiration in comparison to State 3 with increasing temperature. The rate of respiration after addition of rutamycin was near the first State 4 rate at all temperatures indicating that respiration was still "coupled" to phosphorylation. This observation suggests an increase in ATPase activity at the higher temperature as was shown for rat skeletal muscle mitochondria (16). In that system, increasing temperature, especially above 37°, caused an increase

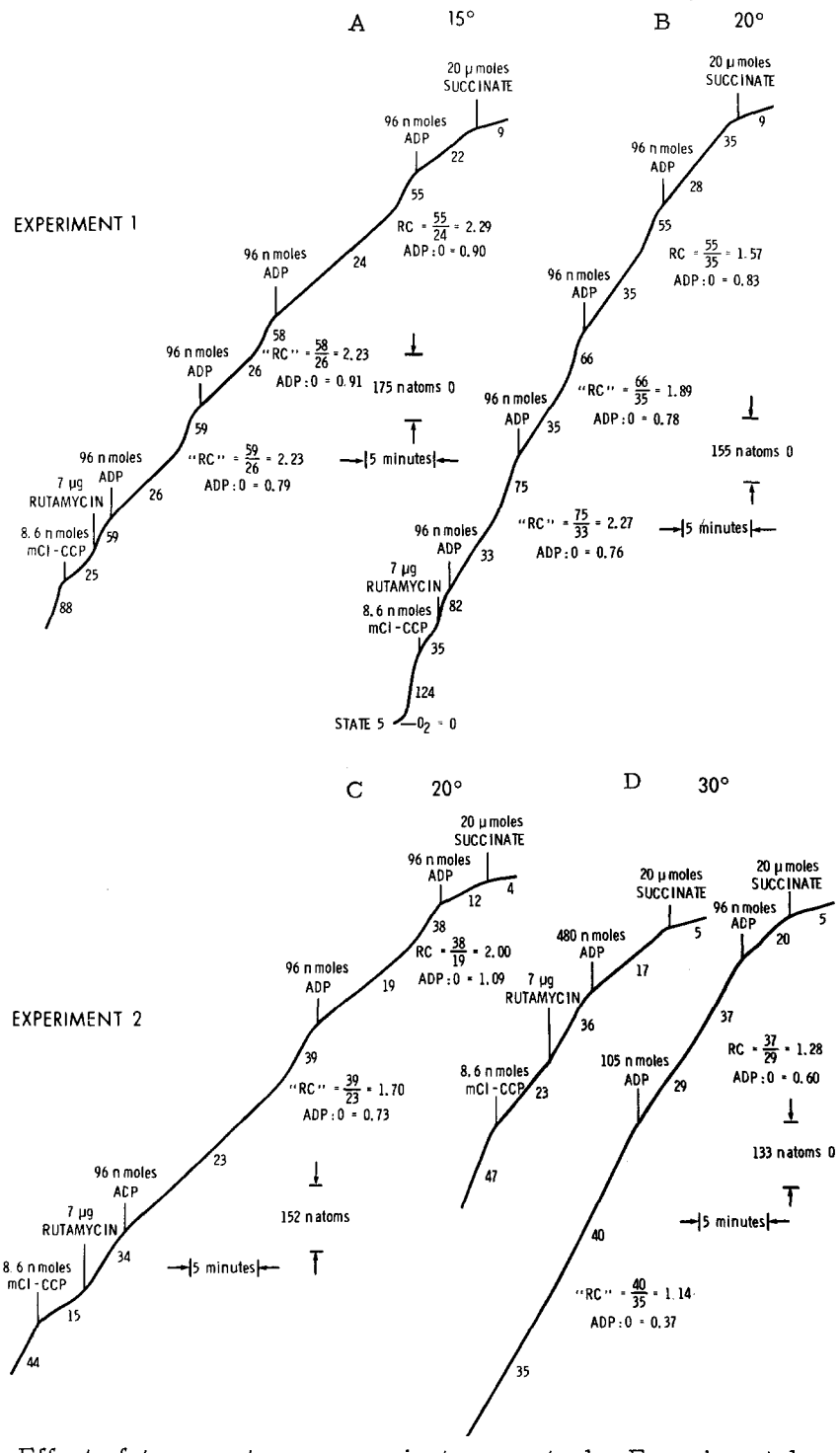

Fig. 3. Effect of temperature on respiratory control. Experimental procedure as described in Fig. 2, except reaction volume was 3.2 ml and total mitochondrial protein was 1.4 mg in experiments 1 and 2 which were different preparations. Reactions were incubated at temperatures indicated. DMF at concentrations used in mCl-CCP and rutamycin additions showed no effect on the reactions.

in oligomycin-sensitive ATPase and a lowering of the RC ratio. Since Crithidia does not grow well above 28°, this phenomenon may be of physiological significance. However, discontinuous step gradients are being investigated as a means of isolating only structurally intact mitochondria free of any contaminating damaged organelles, flagella, and cellular debris which may contribute to ATPase activity. Preliminary results indicate that this technique will be successful, but the yields obtained to date are insufficient for detailed studies. The feasibility of phosphorylation studies in whole cells by techniques such as those described by Hempfling for E. coli (17) are also being considered in relation to determining the significance of this phenomenon. We have noted that whole cell respiration can be stimulated two to four fold by the uncoupler mCl-CCP.

Most preparations contain significant levels of ATPase activity even when assayed at the lower temperatures. Therefore attempts to measure RC and ADP:O ratios with substrates having lower Q_{O_2}'s than succinate were inconclusive because the rate of oxidative phosphorylation in State 3 was unable to compete successfully with ATP breakdown by ATPase. Manometric experiments utilizing a hexokinase-glucose trap were undertaken to characterize the phosphorylating efficiency with other substrates. Typical results are reported in Table I. Electron transport and oxidative phosphorylation with these substrates were similar to succinate in their sensitivity to antimycin A, rutamycin, and mCl-CCP.

NADH alone was oxidized by most preparations with widely variable specific activities. Respiration was fully sensitive to antimycin A and KCN. Lower but significant RC ratios were demonstrable. P:O ratios obtained manometrically have varied from 0.6 to as high as 1.6 and this phosphorylation was sensitive to rutamycin and mCl-CCP. This suggests the presence of partially damaged mitochondria in most preparations rather than a permeability to NADH. Further

TABLE I

MANOMETRIC DETERMINATIONS OF OXIDATIVE PHOSPHORYLATION

Reaction mixture	Oxygen uptake μ atoms	Δ Pi μ moles	P/O
Experiment 1			
complete: succinate (16 mM)	3.74	3.53	0.94
- substrate	0.27	0	0
+ antimycin A (0.8 μg/ml)	0.31	0	0
+ mCl-CCP (3.4×10^{-6} M)	3.77	0	0
+ rutamycin (2.9 μg/ml)	1.79	0	0
Experiment 2			
complete: DL-α-glycerophosphate (66 mM)	3.77	0.96	0.25
- substrate	0.28	0	0
+ antimycin A (0.8 μg/ml)	0.23	0	0
+ mCl-CCP (3.4×10^{-6} M)	3.44	0	0
+ rutamycin (2.9 μg/ml)	2.93	0	0
Experiment 3			
complete: α-ketoglutarate (20 mM)	3.75	4.45	1.19
- substrate	0	0	0
+ antimycin A (0.8 μg/ml)	0.52	0	0
+ mCl-CCP (3.4×10^{-6} M)	2.80	0	0
+ rutamycin (2.9 μg/ml)	1.99	0	0
Experiment 4			
complete: malate (10 mM)	2.43	1.05	0.43
+ NAD^+ (0.4 mM)	3.53	3.55	1.01
complete: pyruvate (10 mM)	0.31	0	0
+ NAD^+ (0.4 mM)	0.64	0	0
complete: malate (10 mM) pyruvate (10 mM) NAD^+ (0.4 mM)	3.97	4.03	1.02

Reaction vessels contained in the main compartment, 0.29 mmoles of MOPS-KOH buffer, pH 7, 0.82 mmoles of sucrose, 15 μmoles of KPO_4, 6.4 μmoles of Mg^{++} acetate, 10 μmoles of ATP, 50 μmoles of glucose, 1.5 mg of hexokinase (Sigma type III), 2.6 mg of BSA, 1.8 μmoles of EGTA, and 1.2, 1.0, 1.0, and 1.4 mg of enzyme protein in experiments 1, 2, 3, and 4, respectively. Substrates and effectors, where indicated, were added from the sidearm at the end of a 10 min. preincubation period at 20°. 0.2 ml of 20% KOH was present in the center well. Final reaction volume was 2.5 ml. When the vessel containing the complete reaction mixture had reached 3-4 μatoms of oxygen uptake, the reactions were terminated by immediate chilling in an ice bath and addition of 1.0 ml of cold 10% TCA. Solvent controls of DMF and ETOH were performed. DMF had no discernible effect; ETOH usually slightly stimulated respiration but did not alter the P:O.

purification of intact organelles is necessary to resolve this problem.

ACKNOWLEDGEMENTS

We should like to thank Drs. J. P. Kusel and B. T. Storey for making their independent work of a similar nature known to us prior to the submission of the manuscript (18). We should also like to thank Drs. R. Cockrell and A. Kaplan for stimulating discussions. This work was supported by Grant AI-03046 from the National Institutes of Health, U.S. Public Health Service.

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