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On Methods of Isolation of Active, Tightly Coupled Mitochondria of Wheat Seedlings[1]

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Abstract. **Tightly coupled mitochondria can be isolated from wheat seedlings 1) if young seedlings are used, 2) if the grinding medium is buffered and contains bovine serum albumin (BSA), 3) if the isolation procedure, including centrifugation is 10 to 11 minutes long. Mitochondria isolated in this manner oxidize α-ketoglutarate, malate and NADH. Respiratory control (R.C.) values are around 6, 3, and 10, respectively. ADP:O values with those substrates are 4.0, 2.9, and 1.7, respectively. Mitochondria are stable for 3 hours. It is suggested that the technique may be used for preparation of mitochondria for comparative studies since activities of these mitochondria are highly reproducible.**

For continuation of studies of regulation of mitochondrial respiration by indoleacetic acid (10) and for studies of mitochondrial heterosis and complementation (5, 11), methods of isolation of mitochondria were desired which would yield mitochondria with high respiratory control by ADP and good P/O ratios. It was also extremely important that the isolated mitochondria be sufficiently stable in order that meaningful and reliable comparative data could be obtained routinely. This would be especially critical in studies of mitochondrial heterosis and complementation since information on these 2 phenomena is based strictly on comparisons of respiratory activity of mitochondria from hybrids with those of the parents of the hybrid analyzed on the same day (11). We decided to study variables in the preparation and the measurement of respiration of mitochondria of wheat seedlings. The variables affecting stability of isolated mitochondria are composition of grinding media, speed and duration of centrifugation, composition of the reaction mixtures and the nature and age of plant tissues (3, 7, 8, 12, 13). Our major interest was to select or develop a technique for the isolation of active mitochondria which would enable us to compare with the least amount of variability many samples of mitochondria in 1 day.

Materials and Methods

Oxidative activity of mitochondria was measured polarographically at 27° with a Clark-type oxygen electrode (Yellow Springs Instrument Company) inserted in a 3 ml lucite chamber. Materials were introduced into the chamber through a small slot and the reaction mixture was stirred by a small magnetic stirrer. Respiratory control (R. C.) values were calculated in accordance with Chance and Williams (1, 2) as the ratio of respiration rate in State 3 (phosphate, oxygen, substrate, and ADP in excess) and respiration rate in State 4 with ADP in limiting amount. An amount of ADP (100–200 μM) was added such that at least 3 control values could be determined before oxygen exhaustion. ADP:O ratios were determined from the amount of oxygen utilized during State 3 responding to a known amount of added ADP. Prior to measurement of activity, mitochondria were thermally equilibrated in the chamber for 3 minutes in the reaction mixture but without added substrate or ADP.

Seedlings of wheat (*Triticum aestivuum*, L.) were grown at 27° in the dark on moistened paper toweling. Six to 8 grams of shoot tips of 2 to 3 day old seedlings were harvested and immediately chilled on crushed ice. All subsequent isolation steps were carried out at 1 to 2° and all glassware and buffers were prechilled. The shoots were homogenized for 40 to 60 seconds in a mortar with 10 ml of grinding medium/gram of tissue. Four grinding media were tested: 1) 0.5 M sucrose, 0.005 M ethylenediamine tetraacetate (EDTA) in 0.067 M potassium phosphate buffer, pH 7.2; 2) same as 1 but with 0.75 mg BSA/ml; 3) 0.5 M sucrose, 0.001 M EDTA in 0.067 M potassium phosphate buffer, pH 7.2 and with 0.75 mg BSA/ml; 4) the grinding and washing media described by Ikuma and Bonner (3).

Centrifugation Procedures. Several centrifugation procedures were used: Procedure W: Following filtration through 4 layers of cheesecloth, the homogenate was distributed in equal amounts into 8 centrifuge tubes. These were centrifuged 5 minutes at 500g and the sediment was discarded. The supernatant was centrifuged 15 minutes at 20,000g;

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the sedimented mitochondria were resuspended in fresh grinding medium or in the washing medium and centrifuged 15 minutes at 20,000*g*. The final pellet was resuspended in 0.5 ml of 0.3 M mannitol. Care was taken to avoid suspending the underlying starch layer. Mitochondria suspended in 0.3 M mannitol were ready for use. Time involved in this procedure was 70 minutes. Procedure X: The first steps were the same as in Procedure W, but the supernatant following the 500*g* centrifugation was centrifuged 10 minutes at 20,000*g*. The sedimented mitochondria were washed with the grinding medium; however, the liquid was added to the centrifuge tubes without suspending the mitochondria. Following the centrifugation for 5 minutes at 20,000*g*, the mitochondria were suspended in 0.3 M mannitol for use. Total time for procedure X was 45 minutes. Procedure Y was similar to X except that the 2 high speed centrifugations were each 5 minutes long at 40,000*g*. Total time for Procedure Y was 30 minutes.

Reaction Mixtures. The next point of interest was with regard to composition of the reaction mixture. Oxidation by mitochondria of 10 mM α-KG (α-ketoglutarate) was studied in 3 different reaction mixtures: Mixture A contained 0.3 M mannitol, 0.01 M KCl, 5 mM $MgCl_2$, 0.01 M potassium phosphate buffer (pH 7.2), 0.01 M tris-HCl buffer (pH 7.2) and 0.75 mg BSA/ml. Mixture B was identical with Mixture A except that tris and BSA were not included. Mixture C was identical to Mixture A and in addition contained 0.33 mg of NAD and 0.01 mg of cytochrome *c*. The final volume of each reaction mixture, after the addition of 0.5 ml of mitochondrial suspension was 3.0 ml. Mitochondrial nitrogen was estimated following determination of mitochondrial protein in BSA-free suspensions by the method of Lowry *et al.* (4). Organic chemicals were obtained from Sigma.

Results and Discussion

The most suitable grinding medium was medium 3, yielding mitochondria with high R. C. and ADP:O values. Mitochondria extracted by medium 4 (3) although giving fairly high R. C. and ADP:O values, are quite variable possibly reflecting the damaging property of this unbuffered medium.

Comparison of procedures of isolation reveals that R. C. and ADP:O ratios increase markedly when isolation time is reduced from 65 to 30 minutes (table I). Mitochondria prepared by Procedure Y were tested in 3 reaction mixtures with α-KG as substrate. Reaction mixture A containing mannitol, tris, potassium phosphate, $MgCl_2$, KCl, and BSA at pH 7.2 was the best of the 3 as judged by R. C. and ADP:O values and the number of cycles of respiration. This reaction mixture was selected as the standard mixture for all experiments.

Table I. *Respiratory Activity of Mitochondria Isolated by 3 Centrifugation Procedures*

Procedures are described in Materials and Methods. The times shown are from grinding to final suspension of isolated mitochondria. Standard reaction mixtures with α-KG as substrate were used. Data are averages of 5 isolations.

	W	X	Y
Isolation time (min)	65	45	30
Total nitrogen in the mitochondrial fraction (mg)	1.20	1.25	0.85
Rate of oxygen uptake in State 3. (μM O_2 per mg N per min)	31.4	56.0	64.6
Rate of oxygen uptake in State 4. (μM O_2 per mg N per min)	13.3	16 5	16.8
R.C. ratio	2.4	3.4	3.8
ADP:O ratio	2.5	2.9	3.0

Using the methods developed thus far, we tested activity of mitochondria utilizing α-KG, α-KG with malonate, NADH and malate (fig 1). Inclusion of malonate with α-KG resulted in higher respiratory control. Although oxidation in State 3 and State 4 were both decreased by malonate, decrease of State 4 was greater, thus yielding higher R. C. values than in oxidation of α-KG without malonate. The ADP:O values for all 4 substrates, while somewhat lower than the generally accepted "theoretical" values, appeared to be satisfactory. During the course of tests of substrates, it became apparent that in working with several samples which were always kept on ice, activity of mitochondria decreased as the experiments progressed on a given day. In other words, among 6 or 8 samples isolated simultaneously and tested for activity at about 20 minute intervals, the last 3 or 4 samples tested dropped in activity. This change in activity would be extremely important in comparative studies since possible real differences in mitochondrial activity would be masked or confounded by the apparent "aging" of the isolated mitochondria. To check this "aging" *in vitro,* we measured activity of mitochondria at various intervals after centrifugation (table II). Both respiratory control and ADP:O ratios decreased as the mitochondria aged on ice. Respiratory control ratio dropped sharply in 75 minutes, primarily because State 4 oxidation increased. The ADP:O ratio remained constant for the first 45 minutes, but decreased thereafter. Therefore, it is obvious that data comparing activities of mitochondria from, for example, different genetic sources or from variously treated plants or plant parts and necessarily analyzed on a given day, would be questionable because of the "aging" of isolated mitochondria. For comparative study, then, mitochondria must be isolated more quickly than described thus far and must maintain high activity for at least 2 to 3 hours.

To this end a method for isolating mitochondria similar to the one described recently by Palmer (9) was used. This procedure involves straining the

Table II. *Effects of Aging of Isolated Mitochondria* in vitro

Experimental procedure: isolation procedure Y and standard reaction mixture. Data are means of 4 experiments.

Time after isolation	O_2 uptake State 3	O_2 uptake State 4	R.C.	ADP:O	Decrease in mito activity R.C.	Decrease in mito activity ADP:O
min	*μM per mg N per min per 3 ml*		*ratio*	*ratio*	%	%
0	75.4	23.3	3.2	2.8	...	...
30	76.3	27.4	2.8	2.9	14	...
45	71.9	28.8	2.5	2.7	23	...
60	85.4	35.7	2.4	1.9	26	31
75	78.5	37.3	2.1	1.9	35	32
90	72.7	36.9	2.0	1.8	39	35
105	76.4	41.0	1.9	1.7	42	40
120	74.9	41.1	1.8	1.6	44	41

Table III. *Summary of Rapid Isolation of Active Mitochondria*

Procedure Z was used in this isolation.

Steps:	Time:
1) Homogenize wheat shoots in grinding buffer in mortar.	45-60 sec.
2) Strain homogenate through 2 layers of nylon fabric (mesh: 50/cm). Distribute filtrate into 6-8 centrifuge tubes.	20-30 sec.
3) Centrifuge tubes at 40,000*g*.	2 min.
4) Wash[1] pellet with grinding buffer, but do not suspend pellet.	1 min.
5) Centrifuge tubes at 20,000*g*.	2 min.
6) Suspend mitochondria in 0.3 M mannitol.	4 min.
Total time:	10-10.5 min.

[1] The washing step, although not used by Palmer (9), is extremely important as it further separates mitochondria from starch.

Table IV. *Oxidative and Phosphorylative Activities of Mitochondria Isolated by Procedures Y (30 Min) and Z (10 Min)*

Tissue and reaction mixture as in table I. Data are averages ± S.E. of 5 experiments.

Procedure	Substrate	O_2 uptake State 3	O_2 uptake State 4	R.C.	ADP:O
		μM per mg N per min per 3 ml		*ratio*	*ratio*
Y	α-Ketoglutarate	69.3 ± 3.1	21.5 ± 1.1	3.2	2.7
Z		36.0 ± 3.5	6.6 ± 1.7	5.4	3.9
Y	α-Ketoglutarate + malonate	50.8 ± 2.6	13.0 ± 1.0	3.9	3.2
Z		45.8 ± 4.5	7.0 ± 1.0	6.4	4.0
Y	Malate	66.5 ± 4.5	29.8 ± 1.5	2.2	2.0
Z		68.6 ± 4.0	21.0 ± 1.5	3.2	2.9
Y	NADH	75.3 ± 6.4	15.7 ± 1 0	4.8	1.2
Z		57.2 ± 5.0	5.7 ± 2.1	10.0	1.7

original homogenate through a nylon fabric, a step introduced by Nobel (6). This manipulation makes the initial slow centrifugation step unnecessary, reducing isolation time (table III) and yielding excellent mitochondria (fig 2). The respiratory control values are increased greatly by this isolation procedure (compare with fig 1) especially in α-KG with malonate and NADH reaction systems. The ADP:O ratios for all cycles of oxidation of α-KG (Traces A and B) are at the "theoretical" maximum of 4. Two cycles of oxidation of malate are at the maximum of 3 while the ADP:O ratios with NADH are greater than those reported (3) (Traces C and D). Those authors also point out that mitochondrial quality is improved by a period of slow speed centrifugation. Mitochondria prepared by our Procedure Z do not appear to be damaged by the fast centrifugation without any intervening slow

spin. Furthermore, mitochondria prepared by this method are quite stable for at least 3 hours. This may be due to the very rapid separation of mitochondria from possible endogenous uncouplers such as various quinones and fatty acids present in the homogenate. It appears that one very important step in preparation of active and stable mitochondria is speed of isolation. Procedure Y had appeared to be satisfactory at the outset of these studies. However, the new improved Procedure Z (table IV) is

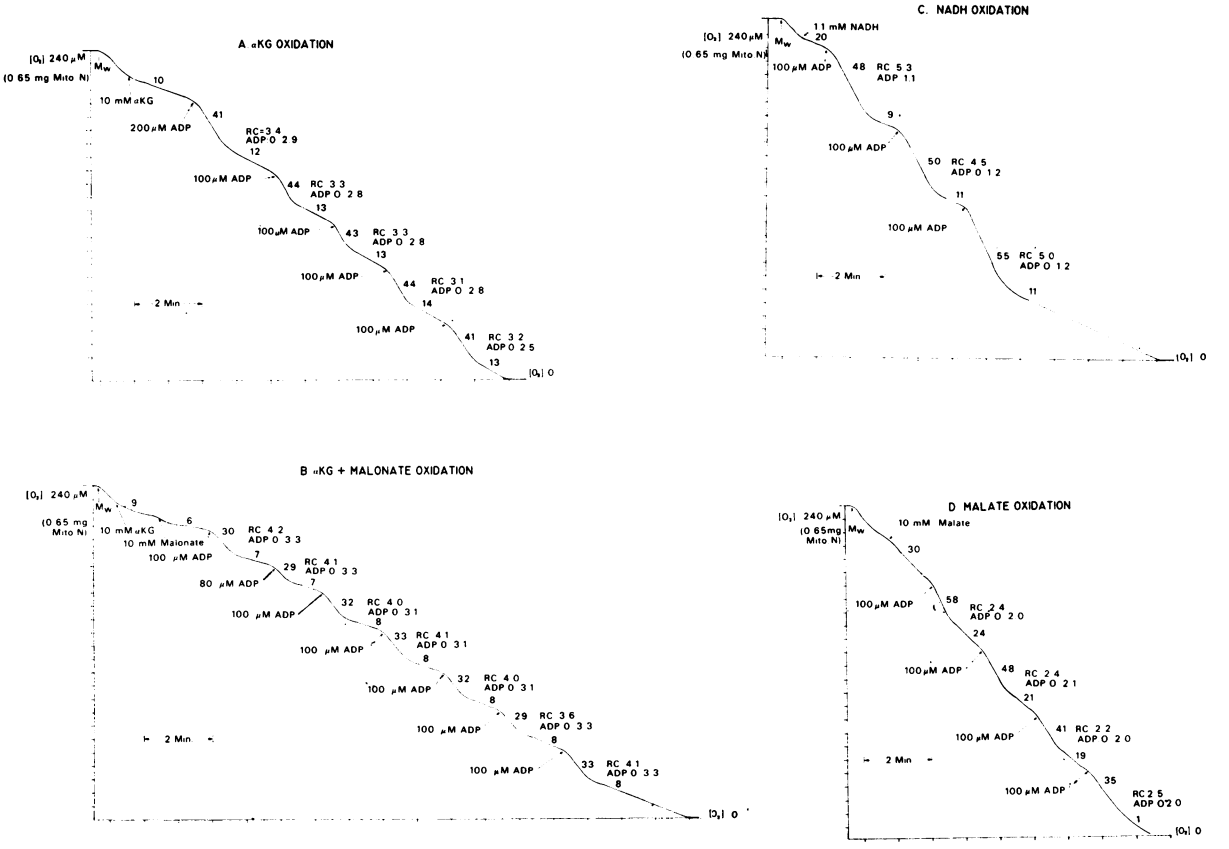

FIG. 1. Patterns of O_2 uptake by wheat shoot tip mitochondria utilizing different substrates. Grinding medium 3 and Procedure Y were used for the isolations. Reaction mixture is given in Materials and Methods. Concentration of substrates, ADP, and mitochondria are shown at the traces. Numbers along the traces are rates of O_2 uptake in μM per min per 3 ml reaction mixture.

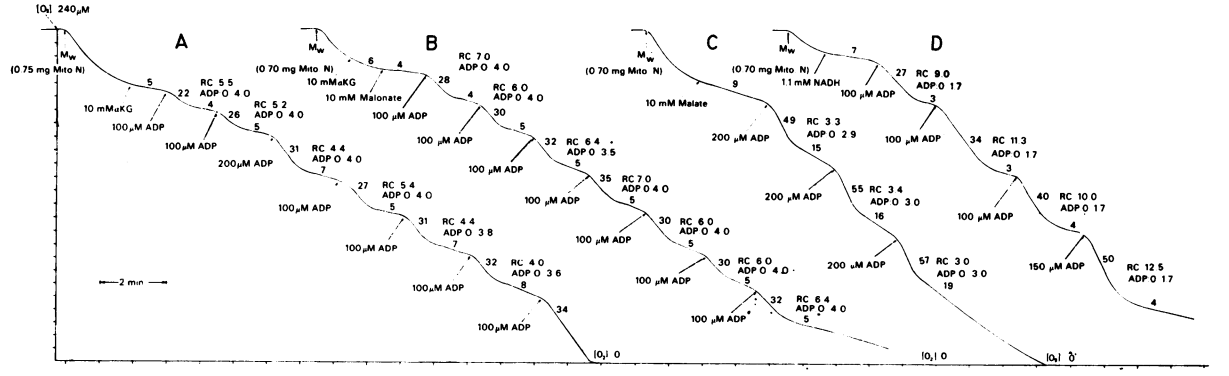

FIG. 2. Pattern of O_2 uptake and phosphorylation by mitochondria from wheat seedlings. Mitochondria were isolated in 10 minutes by Procedure Z. Methods are described in table IV. A) Oxidation of α-KG. B) Oxidation of α-KG with malonate. C) Oxidation of DL-malate. D) Oxidation of NADH. Concentration of added substrate, ADP, and mitochondria is shown at the traces. Numbers along the traces are rates of O_2 uptake in μM per min per 3 ml reaction mixture.

far superior, yielding excellent mitochondria and, most importantly, mitochondria which are stable over a sufficiently long period making it possible to use them with confidence in comparative studies.

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