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Studies on the Respiratory Properties of Mitochondria Isolated from Developing Winter Wheat Seedlings[1]

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ABSTRACT

Mitochondria isolated from shoots of 2 days, light- and dark-grown winter wheat (*Triticum aestivum* L. cv. Rideau) seedlings oxidize α-ketoglutarate and L-malate with good respiratory control and ADP : O ratios. The efficiency of oxidative phosphorylation, and respiratory control are both reduced significantly when succinate or NADH is employed as substrate. Respiratory control values and ADP : O ratios show a general decline in mitochondria from seedlings of increasing age, whether grown in light or dark. In light-grown seedlings, the decrease in respiratory control with aging is due principally to a decrease in the rate of state 3 respiration, while in dark-grown material, the decrease appears to be due mainly to an increased rate of state 4 respiration. In both light- and dark-grown seedlings, oxygen consumption during state 3 respiration is severely inhibited by oligomycin. During state 4 respiration, 2,4-dinitrophenol stimulates oxygen uptake to a level approximately two-thirds the normal ADP-stimulated rate.

The studies of Sarkissian and Srivastava (20, 22) have demonstrated that excellent respiratory control indices and ADP:O ratios greater than the "theoretical" discussed by Lehninger (13) can be obtained from mitochondria of wheat seedlings. They have reported further (25) that respiratory control and ADP:O ratios decline with decreasing age of seedlings, and that these parameters are significantly greater in mitochondria from shoots than from roots or leaves. These investigations have demonstrated that the shoots of young wheat seedlings provide an excellent system for the examination of the respiratory properties of plant mitochondria.

Current studies (18, 23, 24) in our laboratory are focused on structural and biochemical changes associated with the development of cold hardiness in plants. Developing winter wheat seedlings attain a high degree of freezing tolerance when grown at 2 to 4 C (4, 17), and this resistance has been correlated with changes in cell membrane composition during growth at low temperature (3, 4). It was decided, therefore, to use the developing winter wheat seedling system to examine both functional responses of isolated wheat mitochondria and structural changes in mitochondrial membranes to growth at low temperature. This paper describes the respiratory properties of mitochondria isolated from developing winter wheat seedlings grown in light and dark at 21 C, from which the system for subsequent low temperature studies was adapted.

MATERIALS AND METHODS

Seedlings of winter wheat (*Triticum aestivum* L. cv. Rideau) were germinated and grown at 21 C on moist filter paper in dark and in light (8000 lux) for 2, 3, 5, and 7 days. Two to five g of shoots were harvested in a cold room at 2 to 3 C using precooled glassware and media, and all subsequent operations were carried out at this temperature, unless otherwise noted. The shoots were homogenized for 10 to 20 sec in a mortar and pestle, with grinding medium delivered at a uniform rate from a burette by a fine tube. When the first grinding was completed, the mortar contained approximately 8 ml of grinding medium/g of tissue. The homogenate was filtered through two layers of nylon cloth (mesh about 50 μm) into centrifuge tubes. The residue in the nylon mesh was transferred to the mortar and pestle, reground with grinding medium, and refiltered through the nylon. Several grinding media were tested (9, 19, 20), and the most satisfactory was that of Sarkissian and Srivastava (20), composed of 0.5 M sucrose, 1 mM EDTA, 67 mM KH_2PO_4, and 0.75% (w/v) bovine serum albumin at pH 7.2.

Several centrifugation procedures were used initially to isolate mitochondria, including the rapid isolation procedure of Sarkissian and Srivastava (20, 21) and the more conventional procedures of Ikuma and Bonner (9) and Raison and Lyons (19). The procedure finally adopted for this study was a modification of the above methods. After filtering through nylon, the homogenate was centrifuged at 2,000*g* for 5 min, and the resulting supernatant fraction was centrifuged at 20,000*g* for 4 min. The mitochondrial pellet was resuspended in 10 ml of grinding medium, centrifuged at 1,500*g* for 5 min, and the mitochondria sedimented from the supernatant at 8,000*g* for 15 min. The mitochondrial pellet was suspended in 0.5 ml of a suspension medium composed of 0.3 M mannitol, 1 mM EDTA, and 0.1% (w/v) bovine serum albumin at pH 7.2.

Oxygen uptake was measured polarographically at 24 C with a conventional Clark oxygen electrode inserted into a 1.5 ml glass reaction chamber. Materials were introduced into the chamber through a capillary by a syringe, and the reaction mixture was stirred by means of a magnetic stirrer. The reaction mixture was composed of 0.30 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 10 mM tris-HCl buffer, and 0.75% (w/v) bovine serum albumin, adjusted to pH 7.2 at 24 C. Aliquots of mitochondrial preparations were added to the chamber and allowed to equilibrate for 2 min. Sufficient amounts of 0.75 M substrate stock solutions (dissolved in 0.1 M tris, pH 7.2) were added to give a final concentration of 10 mM for α-ketoglutarate, L-malate, and succinate, and 1 mM for

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Table I. *Respiratory Characteristics of Mitochondria Isolated from 2 Day Dark-grown Seedlings by Three Different Methods*

Data are averages ± the standard error of three to five cycles from three experiments.

Isolation Method	Substrate	Oxygen Uptake		Respiratory Control	ADP:O
		State 3	State 4		
		μM/min·mg protein			*ratio*
Sarkissian and Srivastava (20)	L-Malate	72.8 ± 3.0	20.8 ± 1.8	3.5 ± 0.4	2.3 ± 0.1
Ikuma and Bonner (9)	L-Malate	68.0 ± 8.6	12.2 ± 2.1	5.6 ± 0.3	2.3 ± 0.2
Modified	L-Malate	68.9 ± 9.2	10.7 ± 2.0	6.2 ± 0.6	2.4 ± 0.1

Table II. *Respiratory Characteristics of Mitochondria Isolated from Developing Wheat Seedlings Grown in Light and Dark*

Data are averages ± the standard error of three to five cycles from four or five experiments.

Age of Seedlings	Growth Conditions	Substrate	Oxygen Uptake		Respiratory Control	ADP:O
			State 3	State 4		
days			*μM/min·mg protein*			*ratio*
2	Light	α-Ketoglutarate	57.2 ± 7.7	11.9 ± 2.6	4.8 ± 1.9	2.9 ± 0.3
3			44.4 ± 6.1	11.5 ± 1.4	3.9 ± 0.7	3.1 ± 0.3
5			28.5 ± 2.5	12.7 ± 1.1	2.4 ± 0.3	1.9 ± 0.2
7			24.7 ± 3.0	14.9 ± 1.2	1.6 ± 0.1	1.5 ± 0.1
2	Dark	α-Ketoglutarate	73.3 ± 8.5	11.3 ± 3.0	6.5 ± 1.2	3.0 ± 0.1
3			59.2 ± 8.9	12.5 ± 1.6	4.8 ± 1.2	2.9 ± 0.3
5			48.6 ± 2.5	16.2 ± 2.8	3.0 ± 0.6	2.3 ± 0.2
7			58.4 ± 5.8	22.5 ± 3.4	2.6 ± 0.2	2.0 ± 0.0
2	Light	L-Malate	61.9 ± 12.5	9.6 ± 2.2	6.4 ± 2.0	2.1 ± 0.0
3			56.5 ± 7.5	14.2 ± 2.1	4.0 ± 0.7	2.3 ± 0.2
5			35.8 ± 3.7	14.3 ± 2.1	2.5 ± 0.5	1.9 ± 0.1
7			22.7 ± 4.0	13.4 ± 1.7	1.7 ± 0.1	1.4 ± 0.2
2	Dark	L-Malate	66.5 ± 9.3	10.4 ± 1.9	6.3 ± 1.4	2.2 ± 0.0
3			73.6 ± 16.0	16.3 ± 5.1	4.8 ± 1.6	2.3 ± 0.2
5			53.4 ± 3.5	20.5 ± 2.1	2.6 ± 0.5	1.8 ± 0.2
7			46.9 ± 8.8	26.1 ± 3.8	1.8 ± 0.2	1.7 ± 0.2

NADH. State 3 respiration was initiated by addition of 10 or 20 μl of ADP to give a final concentration of 50 or 100 μM. The effect of DNP[2] and oligomycin on the rates of state 3 and state 4 respiration was examined by addition of DNP and oligomycin to final concentrations of 0.1 mM and 6 μg/ml, respectively. State 3 and state 4 respiration rates were calculated from recorder tracings on the basis of 240 μM O_2 in aerated medium (2). All rates of O_2 consumption are expressed as μM/min·mg protein.

Organic chemicals used in this study were purchased either from Schwartz/Mann Ltd. or Sigma Chemical Company. All inorganic reagents were of analytical reagent grade, solutions were prepared in double-distilled water, and the pH of all substrates were adjusted to 7.2. The concentration of ADP was determined optically at 260 nm on the basis of a millimolar extinction coefficient of 15.4. Mitochondrial protein was determined in bovine serum albumin-free suspensions by the method of Lowry *et al.* (15), and by microkjeldahl (26).

RESULTS

Respiratory activities of mitochondria isolated from 2 day dark-grown Rideau winter wheat seedlings by three different methods are shown in Table I. The rate of state 3 O_2 consumption is similar for the three methods, but state 4 respiration is greater in mitochondria isolated by the rapid procedure of Sarkissian and Srivastava (20) than by either the procedure of Ikuma and Bonner (9) or the modified procedure subsequently adopted for this study. Hence, greater respiratory control values are obtained from the latter two procedures. ADP:O ratios are similar for mitochondria isolated by the three methods.

The possible effect of microbial contamination on respiratory activity was tested by isolating mitochondria in the presence of tetracyclin, a potent inhibitor of microbial respiration, and by isolating mitochondria under standard conditions and then inactivating the mitochondrial respiratory system by incubation at 37 C for 10 min (22). Oxygen uptake is completely abolished by incubation at 37 C but is unaffected by isolation in the presence of tetracyclin, indicating that microbial respiration does not contribute significantly to the observed respiratory rates.

Respiratory control values and ADP:O ratios are generally highest in mitochondria isolated from 2 day seedlings whether grown in light or dark (Table II). Since our current studies on low temperature acclimation involve the use of dark-grown seedlings, it was decided to examine the respiratory properties

[2] Abbreviation: DNP: 2,4-dinitrophenol.

of various substrates on the two day dark grown material. The activity of mitochondria isolated from shoots of 2 day dark-grown seedlings utilizing different substrates is shown in Figure 1. The mitochondria appear to be in good condition, as indicated by relatively high rates of state 3 (ADP-stimulated) respiration, good respiratory control values (6–8), and several cycles of controlled respiration for oxidation of both α-ketoglutarate and malate. ADP:O ratios of approximately 3.0 and 2.3 for α-ketoglutarate and L-malate, respectively, indicate a relatively high efficiency of oxidative phosphorylation. A rapid rate of O_2 consumption during state 3 respiration is observed also utilizing NADH and succinate as substrates, but state 4 (ADP-exhausted) respiration is much greater than observed for either α-ketoglutarate or malate, resulting in lowered respiratory control values. The efficiency of oxidative phosphorylation using NADH and succinate is also reduced relative to that observed for α-ketoglutarate and malate. When mitochondria are isolated in a medium previously described by Srivastava and Sarkissian (25) specifically for examination of succinate oxidation, a significant increase in respiratory control is observed, but respiratory control values increased only to 2.3 to 2.5.

The effect on respiratory activity of oligomycin and of DNP is shown in Figures 2 and 3. The addition of oligomycin results in nearly total inhibition of state 3 respiration utilizing either α-ketoglutarate (Fig. 2A) or malate (Fig. 3A) as substrate. It also inhibits the slightly increased rate of state 4 respiration which is observed approximately 0.5 to 1 min after ADP exhaustion, during oxidation of malate (Fig. 3B). The addition of DNP during state 4, or oligomycin-inhibited state 3 respiration (Figs. 2A and 3A) results in a marked increase in the rate of O_2 consumption with both substrates. However, DNP-stimulated respiration using either α-ketoglutarate or malate is only 65 to 75% of the normal tightly coupled ADP-stimulated rate.

The addition of oligomycin, followed by DNP, during state 4 oxidation of malate results in an enhancement of the rate of O_2 consumption (Fig. 3B) comparable to that observed when DNP was added during oligomycin-inhibited state 3 respiration (Fig. 3A). In contrast, no significant change in the rate of respiration is observed when DNP is added in the presence of oligomycin during state 4 oxidation of α-ketoglutarate (Fig. 2B). However, if ADP is added to the preparation after the addition of oligomycin, the further addition of DNP results in the increased rate of oxygen consumption (Fig. 2C). Similar results are obtained if oligomycin, DNP, and then ADP are added sequentially during state 4 oxidation of α-ketoglutarate. The addition of AMP under these conditions also results in an increase in the rate of respiration comparable to that observed with ADP.

The respiratory characteristics of mitochondria isolated from shoots of developing winter wheat seedlings grown in light and dark are presented in Table II. A general decline in respiratory control values and in ADP:O ratios is observed for

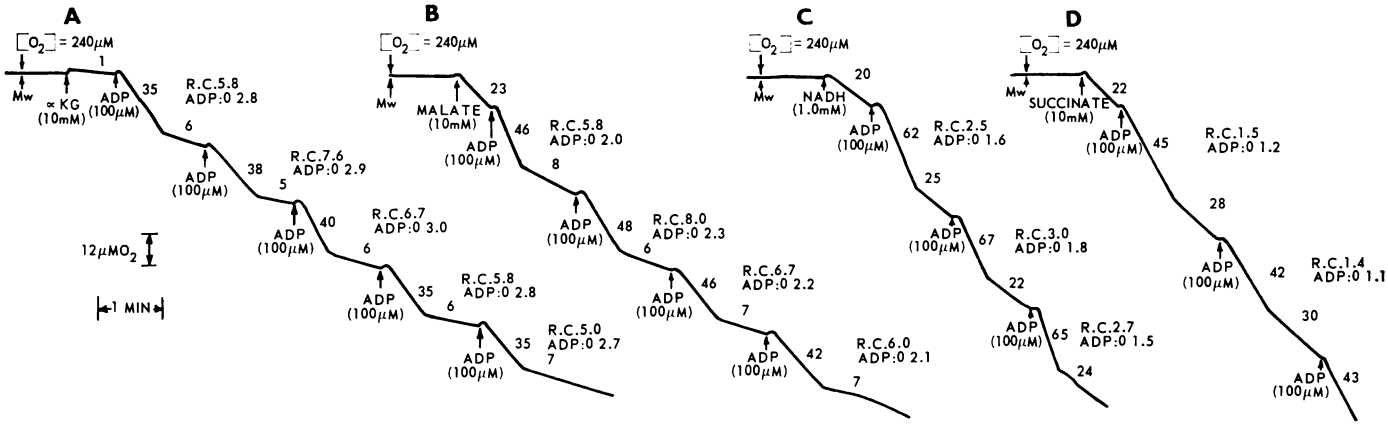

FIG. 1. Polarograph traces of O_2 uptake by mitochondria isolated from shoots of 2 day dark-grown wheat seedlings by the modified method utilizing substrates: α-ketoglutarate (A), L-malate (B), NADH (C), and succinate (D). Numbers along the traces are rates of O_2 uptake in μM/min·1.5 ml. Concentrations of reactants are final concentrations in the 1.5-ml reaction mixture. Mw: addition of mitochondria containing approximately 0.50 mg mitochondrial protein; R.C.: respiratory control.

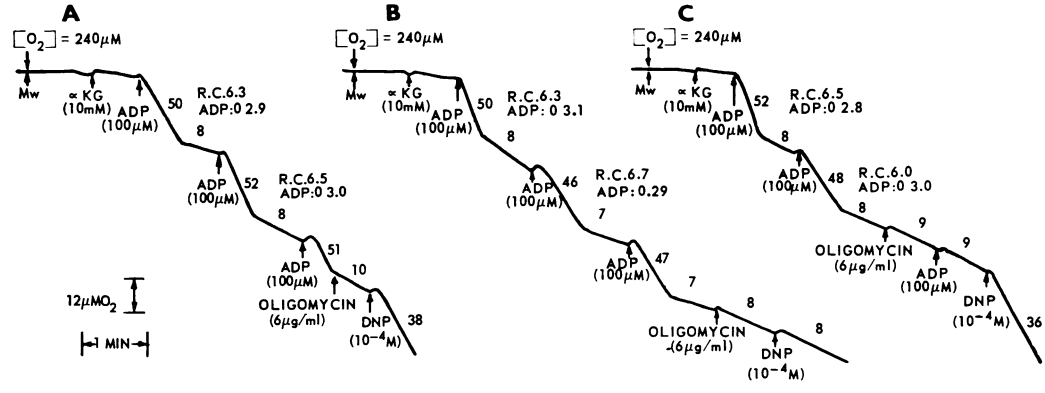

FIG. 2. Polarograph traces showing the effect of oligomycin and DNP on O_2 uptake of wheat mitochondria utilizing α-ketoglutarate as substrate. A: Oligomycin added during state 3 respiration, followed by DNP; B: oligomycin added during state 4 respiration followed by DNP; C: oligomycin added during state 4 respiration, followed by ADP and then DNP. Other conditions were as indicated in Fig. 1.

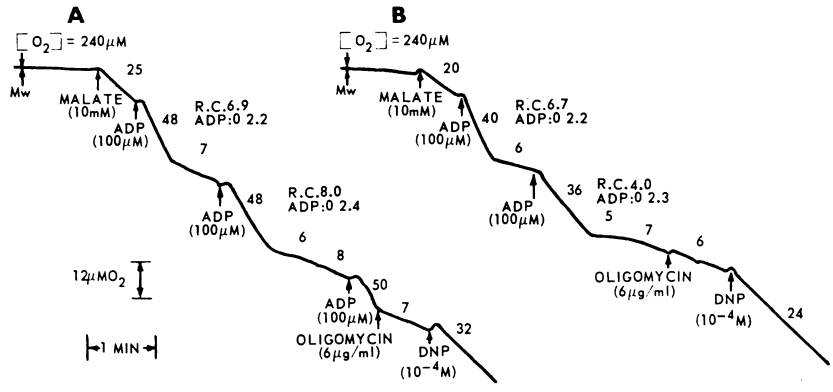

FIG. 3. Polarographic traces showing the effect of oligomycin and DNP on O_2 uptake of wheat mitochondria utilizing L-malate as substrate. A: Oligomycin added during state 3 respiration, followed by DNP, B: oligomycin added during state 4 respiration, followed by DNP. Other conditions were as indicated in Fig. 1.

Table III. *Protein Content of Mitochondria Isolated from Developing Wheat Seedlings Grown in Light and Dark*

Data are averages ± the standard error of three determinations each by the methods of Lowry *et al.* (15).

Age of Seedlings	Protein Content	
	Light-grown	Dark-grown
days	*mg/g fresh weight*	
2	1.17 ± 0.03	0.98 ± 0.06
3	0.68 ± 0.04	0.52 ± 0.05
5	0.74 ± 0.02	0.33 ± 0.01
7	0.68 ± 0.02	0.24 ± 0.01

mitochondria isolated from aging seedlings grown either in light or dark, utilizing either α-ketoglutarate or malate as substrate. Respiratory control values are highest in mitochondria from 2 day seedlings and decline steadily through 7 days. ADP:O ratios are similar for mitochondria from 2 and 3 day seedlings, but decrease significantly at 5 and 7 days.

The change in mitochondrial protein during the early stages of development of Rideau winter wheat grown in light and dark is shown in Table III. The level of protein is slightly higher in the light-grown seedlings at 2 days, and decreases by nearly 50% in both light- and dark-grown seedlings at 3 days. In dark-grown material, further decreases are observed in mitochondria from 5 and 7 day seedlings, while in light-grown material, protein content remains relatively constant throughout the 7 days. The rapid decrease in the level of mitochondrial protein during the early stages of development emphasizes the importance of having seedlings at exactly the same stage of development when comparing respiratory properties of mitochondria from different varieties or of the same variety grown under different conditions.

DISCUSSION

The degree of respiratory control and the efficiency of oxidative phosphorylation (ADP:O ratio) are generally considered to be critical factors for evaluation of the quality of isolated mitochondrial preparations. Previous reports (8–10, 20) have indicated that respiratory properties of mitochondria are greatly influenced by the method of isolation and by the presence of inhibitory substances. In the current study, three methods for the isolation of mitochondria were examined. The lower respiratory control values obtained using the rapid isolation procedure (12–15 min) of Sarkissian and Srivastava (20), rather than a conventional method (9) or the modified method, are at variance with results previously reported for the rapid isolation method. Sarkissian and Srivastava demonstrated that respiratory control increased as the time required for isolation of the mitochondria decreased, whereas in this study respiratory control was higher in mitochondria isolated by both a conventional method (60–65 min) and the modified method (35–40 min). Hence, it was decided to use the modified isolation procedure for the remainder of this study, since it yielded mitochondria which exhibited greatest respiratory control and ADP:O ratios.

The rate and efficiency of utilization of different substrates by plant mitochondria may vary (9, 25), depending on plant source and method of isolation. The results presented in Figure 1 indicate that α-ketoglutarate, malate, and succinate are oxidized at approximately the same rate during state 3 respiration, whereas NADH is oxidized at a somewhat higher rate. However, respiratory control ratios are significantly higher during oxidation of α-ketoglutarate and malate than with NADH or succinate (Fig. 1). Studies of Sarkissian and Srivastava (22) on wheat mitochondria using a different isolation method indicated that respiratory control is greater utilizing NADH or α-ketoglutarate as substrates than with malate or succinate.

Mitochondria prepared by the modified method used in this study possess a very low endogenous rate of respiration (Fig. 1). The addition of α-ketoglutarate to the preparations results in a very small increase in the respiratory rate, whereas the addition of malate, NADH, or succinate greatly enhances the rate of oxygen consumption, even in the absence of exogenous ADP. Similar results have been reported for mitochondria for avocado fruit (27), where the low initial rate of α-ketoglutarate oxidation in the absence of ADP has been attributed to the strong coupling between oxidation and phosphorylation at the substrate level of α-ketoglutarate oxidation. This is substantiated by the higher ADP:O ratios obtained with α-ketoglutarate as substrate. Other studies on mitochondria from wheat (22), mung beans (9), and tomato fruit (10) have indicated that results, both similar and in contrast to those obtained in this study, can be obtained using the same substrates. It is felt, therefore, that the results reported in this paper support the view that mitochondria from various plant sources prepared by different isolation techniques may oxidize the various Krebs cycle substrates at different rates (25).

The results obtained from investigation of the effects of oligomycin and DNP on respiration are generally in accord with those previously reported (5, 11, 22, 27) for plant mito-

chondria. The addition of oligomycin during state 3 oxidation of both α-ketoglutarate and malate results in nearly complete inhibition of ADP-stimulated respiration, as would be expected, based on its known role as an effective respiratory-chain phosphorylation inhibitor (12). Dinitrophenol uncouples phosphorylation during State 4 oxidation, resulting in a markedly enhanced rate of oxidation of both α-ketoglutarate and malate. However, uncoupling is not complete in this system, since, as previously reported (22), measurable respiratory control values and ADP:O ratios are still obtained in the presence of 0.1 mM DNP. This concentration of DNP has been shown to almost completely uncouple oxidation from phosphorylation in animal mitochondria (14).

The differential response of the substrates α-ketoglutarate and malate to the addition of both oligomycin and DNP during state 4 respiration is not clearly understood at present. DNP appears to uncouple electron transport phosphorylation from oxidation when added during oligomycin-inhibited state 3 oxidation of both α-ketoglutarate and malate, or when oligomycin has been added during state 4 oxidation of malate, but not when oligomycin has been added during state 4 oxidation of α-ketoglutarate. Inhibition under the latter conditions, however, is released by the addition of ADP or AMP, but not by ATP as has been reported (5) during succinate oxidation by tomato fruit mitochondria. Further studies currently being conducted in an attempt to clarify the differential effects of antibiotics and nucleotides on the rates of oxidation of Krebs cycle substrates by winter wheat mitochondria will be reported in a later paper.

The decline in respiratory control values and ADP:O ratios observed with increasing age of the seedlings from which the mitochondria were isolated (Table II) are generally in accord with those previously reported for plant mitochondria. However, in the current study, the decline in respiratory control of mitochondria from dark-grown material appears to be due principally to increased state 4 respiration, whereas a previous study on dark-grown wheat (25) demonstrated increased rates of both state 3 and state 4 oxidation of α-ketoglutarate with increasing age of seedlings. Other reports on castor bean and maize (1, 6, 7) indicate that respiratory activity generally increases during the first few days of germination but then declines by the 4th to 5th day. In light-grown material (Table II), a gradual decline in state 3 respiratory activity is observed from 2 through 7 days. It appears, therefore, that although respiratory rates may vary depending on plant source and method of isolation of mitochondria, respiratory control and efficiency of phosphorylation consistently decline with increasing age of seedlings. It was therefore decided to use 2 to 2.5 day dark-grown seedlings to examine structural and functional responses of wheat mitochondria to growth at low temperature (16).

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