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Mitochondrial Longevity *In Vitro*: The Retention of Respiratory Control*

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Abstract. Isolated mitochondria were maintained metabolically active and coupled, i.e., capable of respiratory control, for several hours at 25°C. An increase in respiratory control generally occurred during the first 4–8 hr of incubation followed thereafter by a decline. Longevity is a dynamic function as evidenced by its dependency on substrate and cofactors, thiamine pyrophosphate, in particular. Magnesium was also essential to longevity; coenzyme A, bovine serum albumin, and reducing agents were not. The findings are discussed in terms of cytoplasmic-mitochondrial interrelationships and mitochondrial autonomy.

Introduction. The quasi-autonomous nature of mitochondria is evidenced, in part, by their ability to conserve metabolically derived energy and their capacity to synthesize protein or other structural components *in vitro*.[1] However, the linearity of these functions has seldom been shown to exceed 1 or 2 hr[2] and the resultant data, while highly valuable for point-in-time assessments of the organelles, can only be related to autonomy by extrapolation to *in vivo* conditions. More direct indications of autonomy would accrue if specific *in vitro* functions were shown to be essential for the maintenance of mitochondrial integrity and ultimately, for mitochondrial response to perturbations. Toward this goal we report that isolated mitochondria can remain active and phosphorylating for periods of up to 20 hr at 25°C.

Materials and Methods. Mitochondria were isolated from Bosc and Bartlett pear fruit (*Pyrus communis* L.), and washed once, utilizing methods previously described.[3] For survival at temperatures above 0°C mitochondria (1–3 mg protein) and 3 ml of reaction mixture (as defined in Fig. 1) were placed in 20-ml beakers, covered with parafilm, and then shaken at 120 cycles/min in a 25°C water bath. The same reaction mixture and a Clark oxygen electrode[4] were used for the polarographic assay of oxygen consumption and determination of respiratory control ratios. The latter, a measure of the degree of coupling between oxidative and phosphorylative reactions, is defined as the rate of oxygen consumption in the presence of ADP (state 3), divided by the rate in the absence of ADP (state 4). Mitochondrial protein was determined with a modified Lowry procedure[5] utilizing bovine serum albumin as a standard. For bacterial counts, appropriately diluted mitochondrial suspensions were plated on nutrient agar.

Results. Oxygen consumption by mitochondria previously incubated at 25°C for different periods of time is shown in Figure 1. An increased respiratory rate in response to stoichiometric quantities of ADP (state 3) is clearly evident for mitochondria incubated 2, 8, and 16 hr at 25°C. With this particular

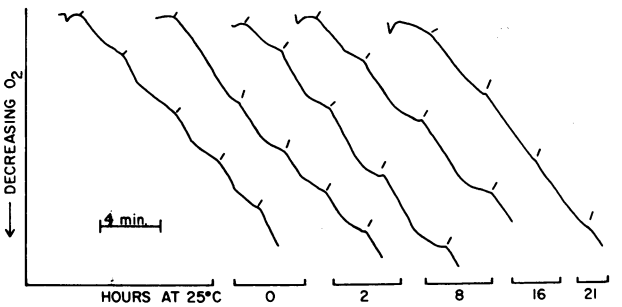

FIG. 1.—Oxygen consumption by pear mitochondria previously incubated at 25°C for the indicated number of hours. Repeated additions of 0.2–0.3 μmole ADP are indicated by the short slant lines. Incubation and reaction mixture: 1.5 mmol sucrose, 60 μmol P_i (pH 6.8), 30 μmol α-ketoglutarate, 0.03 μmol DPN, 0.1 μmol TPP, 3 μmol $MgCl_2$, 0.013 μmol CoA, 3 mg bovine serum albumin. Final volume: 3 ml + 0.02 to 0.03 ml with each addition of ADP. Assay temperature: 25°C.

mitochondrial preparation only a trace of respiratory control was evident after 21 hr although the rate of oxygen consumption remained relatively high.

Several laboratories[6–8] have reported slight increases in mitochondrial respiratory control with each subsequent addition of ADP during a standard assay lasting 15–30 min. The same phenomenon is exhibited as a very distinct increase in respiratory control during long-term incubations, e.g. the 8-hr trace (Fig. 1) and the respiratory control values with 1 mM Mg (Fig. 3).

To assess the requirements for longevity, specific components were omitted from the reaction mixture during incubation and then added just before the polarographic assay. Results of a typical experiment are shown in Figure 2. The absence of substrate (αKG) did not significantly affect the mitochondria for the first 3 hr. However, after 9 hr the mitochondria were completely inactive. Whether the need for metabolically derived energy develops only after a period of incubation or whether the requirement is initially met by endogenous substrates was not discerned. A requirement for thiamine pyrophosphate is manifested after 3 hr at 25°C. DPN was also important to longevity, although some respiratory control remained after 9 hr incubation in the absence of the cofactor.

When plotted against absolute oxygen concentration, as in Figure 2, the initial polarographic readings are roughly proportional to the relative oxygen tension in the incubation medium. With a complete reaction mixture and hence most rapid oxidation rate, the oxygen tension in the incubation medium equilibrated at approximately 60% of saturation. After 3 hr in the absence of thiamine pyrophosphate the mitochondria were almost totally inactive and the oxygen tension again approached saturation. The same conditions prevailed after 9 hr in the absence of DPN. However, lack of each factor must have resulted in a unique form of damage for the inactivity was reversible in the case of DPN, partially reversible for thiamine pyrophosphate, but permanent for αKG. Thus, the absence of αKG, thiamine pyrophosphate, or DPN affects the longevity of mitochondria in diverse ways, manifested by differences in (*a*) oxidative activity during incubation; (*b*) the time when stress is first evident; and (*c*) the reversi-

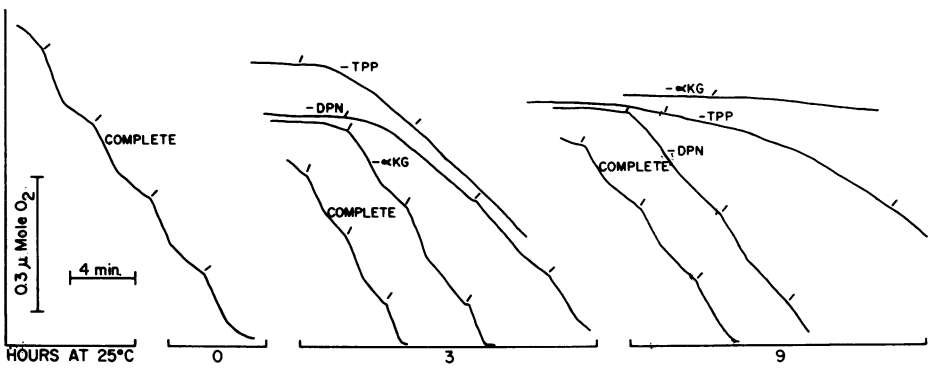

FIG. 2.—Respiratory rates of pear mitochondria before and after a 3- or 9-hr incubation in either complete reaction mixture or reaction mixture deficient in the indicated component. The omitted component was added just before polarographic assay. Other assay conditions as given in Figure 1. Incubation and polarographic assay at 25°C.

bility of the damage. For quantitative comparisons the oxidative rates and respiratory control ratios for the mitochondria in this experiment are recorded in Table 1.

A magnesium requirement for mitochondrial longevity is indicated by the data in Figure 3. Omission of magnesium had initially little effect on respiratory control but did suppress the rise in control during prolonged incubation. One mM magnesium appeared optimal, a higher concentration (10 mM) resulted in an immediate suppression of respiratory control. In contrast to the requirement for magnesium, neither coenzyme A, bovine serum albumin, nor catalytic amounts (0.2–0.5 μmol) of ADP were found essential for longevity. Reducing agents such as cysteine or dithiothreitol also had little or very limited beneficial effect and were harmful at higher (10^{-3} M) concentrations.

Predictably, extended incubation of considerably less than sterile isolates led to bacterial proliferation, e.g., 10^{-8} bacteria/ml after 21 hr. As suggested by Leaver and Edleman,[9] chloramphenicol at 50 μg/ml effectively suppressed bacterial growth. Moreover, the presence of chloramphenicol did not significantly affect the long-term maintenance of respiratory control (Table 2). Thus, it

TABLE 1. *Substrate and cofactor requirements for mitochondrial longevity at 25°C.*

Incubation (hr)	Incubation* medium	Q_{O_2} (N)† (state 3)	Respiratory control ratio
0	Complete	332	3.6
3	Complete	310	2.1
	-αKg	133	1.2
	-DPN	310	2.0
	-TPP	164	1.7
9	Complete	255	1.9
	-αKG	0	...
	-DPN	188	1.7
	-TPP	86	...

* The missing component was added prior to polarographic assay and oxidative rates taken at the point of maximum recovery.

† μl O_2 hr^{-1} mg^{-1} mitochondrial nitrogen.

TABLE 2. *Effects of chloramphenicol and cycloheximide on mitochondrial longevity at 25°C.*

Incubation mixture	Respiratory Control Ratio (Time-hr) 0	2	4	8	20
Control	6.9	3.2	3.1	3.4	1.4
+ Chloramphenicol (50 μg/ml)	3.8	3.1	2.7	2.4	2.0
Control	3.0	3.0	3.3	1.9	...
+ Cycloheximide (200 μg/ml)	3.5	3.1	5.1	2.3	...

appears that continued synthesis of mitochondrial protein may not be essential for longevity. Cycloheximide, thought not to inhibit mitochondrial protein synthesis[2] but having diverse effects on plant cell metabolism,[10] actually appears to enhance the respiratory control of mitochondria both immediately, as previously noted,[11] and during extended incubation (Table 2).

Discussion. Our studies indicate that mitochondria will survive and maintain respiratory control while incubated for several hours at 25°C. Although examined more extensively with mitochondria from pear fruit, longevity has also been observed with avocado mitochondria and presumably is a general phenomenon among the organelles.

Mitochondrial longevity is dependent on vital functions as evidenced by the need for substrate and certain cofactors. An underlying dynamic function is also implicit in the transient increase in respiratory control which generally reaches its maximum between the fourth and eighth hours of incubation.

Some general comments regarding these experiments reflect upon the nature of mitochondrial longevity. Though a preponderant number of mitochondrial preparations maintained respiratory control while incubated at room temperature, the longevity varied from a few (2–3) hours to as long as 24 hr. Moreover, the transient increase in respiratory control, often quite marked, was at other times minimal or completely absent. Untested or undefined parameters of isolation and incubation may well affect longevity. Methods of incubation that permit better control of aeration, constant levels of substrate, and removal of metabolic products should enhance longevity. Various dialysis techniques have been tested with these objectives in mind but none has thus far proven successful.

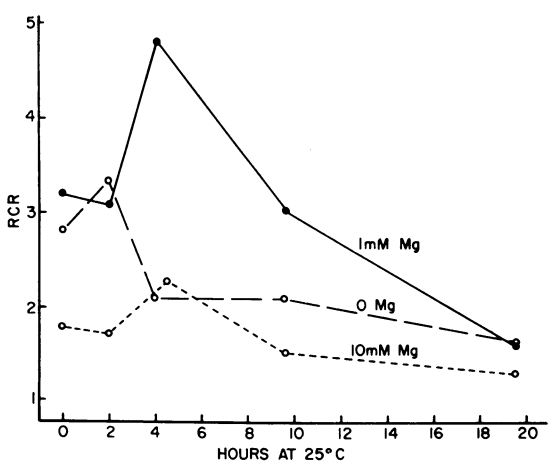

FIG. 3.—The effects of Mg on the maintenance of respiratory control by isolated pear mitochondria. The indicated $MgCl_2$ concentrations prevailed throughout the incubation period and subsequent polarographic assay. RCR: respiratory control ratio.

Preliminary data do indicate that substitution of *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid for phosphate buffer in the isolation and washing media may enhance longevity.

It is known that mitochondria, *in vivo*, will vary in appearance and numbers as a result of physiological stress. Ionizing radiation, for example, will cause a loss of mitochondrial protein and uncoupling with subsequent quantitative and functional recovery dependent upon the extent of initial damage and physiological age of the cells.[12] To the extent that long-lived, isolated mitochondria stimulate mitochondrial functions *in vivo*, some variability in their survival may be expected. Conversely, longer-lived organelles should be useful in assessing the equivalence, or difference, between the functions of isolated mitochondria and those in their natural environment. As emphasized by Hall and Palmer,[13] such an assessment is vital to a further understanding of the interrelationship between mitochondria and cytoplasm or alternatively, the degree of mitochondrial autonomy.

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