

# Ripening and *in Vitro* Retention of Respiratory Control by Avocado and Pear Mitochondria

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## ABSTRACT

The retention of respiratory control ("survival") by mitochondria held at 25 C was studied in relation to the ripening of two varieties of avocado (*Persea americana* Mill. var. 'Fuerte' and 'Hass') and one variety of pear (*Pyrus communis* L. var. 'Bartlett') fruit. The survival of avocado mitochondria increased from 8 to 10 hours when isolated from unripe, preclimacteric fruit, to 48 hours when isolated from fully ripe, postclimacteric fruits. Although rates of  $\alpha$ -ketoglutarate oxidation, respiratory control, and ADP/O decreased somewhat in the postclimacteric phase, survival *per se* was not affected. Pear mitochondria survived for more than 30 hours regardless of the physiological age of the source.

Exposure of postclimacteric avocado mitochondria to a preclimacteric supernatant fraction curtailed their survival. The harmful effect of some unknown substance(s) in the preclimacteric avocado supernatant fraction was confirmed by utilizing pear mitochondria as an independent test system.

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The absence, or near absence, of ultrastructural change in the mitochondria of ripening pear (1), apple (17), and tomato (9) fruits plus the persistent capacity of mitochondria to oxidize substrates and demonstrate respiratory control throughout the climacteric of many fruits, including avocado (10, 14), tomato (8), apple (11-13), and banana (7) attest to the fact that mitochondria in senescing fruit cells remain functionally sound. Nonetheless, Romani *et al.* (26) discerned a ripening, age-related change in the mitochondrial system based on the observation that mitochondria in climacteric pears can neither withstand nor recover from doses of ionizing radiation that are tolerated by mitochondria in preclimacteric fruit.

It has since been shown that isolated pear and avocado mitochondria can maintain RC<sup>1</sup> at 25 C for several hr (23, 24). In analogy to experiments with long lived chloroplasts (6, 18), the maintenance of RC at 25 C has been referred to (24) as "survival." It seems reasonable to assume that survival is a more critical index of organelle integrity than a single point-in-time assessment of RC and should be helpful in understanding the status of mitochondria in ripening fruit. In the present study, the possible relationship between the capacity of mitochondria to survive *in vitro* and fruit ripening and senescence was investigated.

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<sup>1</sup> Abbreviations: RC: respiratory control; RCR: respiratory control ratios;  $\alpha$ -KG:  $\alpha$ -ketoglutarate.

## MATERIALS AND METHODS

**Fruits.** 'Fuerte' and 'Hass' avocados (*Persea americana* Mill.) were obtained from the University of California South Coast Field Station, where they were harvested and immediately shipped to Davis. The fruits were placed at 5 C upon arrival. Preclimacteric 'Bartlett' pears (*Pyrus communis* L.) were obtained from orchards in Sacramento and Lake Counties, California. The pears were held at 0 C prior to ripening and the isolation of mitochondria.

**Preparation of Samples and Measurement of Respiration Rates.** Representative samples of avocados, selected for uniform size and weight, were placed in respiration jars and ripened at 15 C. Pears were ripened in a similar manner at 20 C. Selection of ripening temperatures was based on preliminary studies in which the time required for ripening was sufficient to allow for several mitochondrial isolations at points along the climacteric. The Claypool-Keefer (4) method was used to measure fruit respiration.

**Isolation of Mitochondria.** Mitochondria were isolated from avocado fruits utilizing methods derived from prior studies by Lance *et al.* (14) and Hobson *et al.* (10), and described in detail by Romani and Özelkök (24). The soft postclimacteric avocado tissues were easily disrupted when pressed through a stainless steel screen (25) positioned in the isolation medium (0.25 M sucrose; 50 mM K phosphate, pH 7.2; 0.1% BSA; 5 mM EDTA; 5 mM  $\beta$ -mercaptoethanol; 0.2% PVP). The hard tissues of preclimacteric avocados were macerated by using a porcelain hand grater submerged in the isolation medium. A ratio of 1:3 (w/v), tissue to isolation medium, was used. After sedimentation at 14,000g the mitochondria were dispersed in wash medium (0.25 M sucrose; 50 mM K phosphate, pH 7.2; 0.1% BSA; 5 mM  $\beta$ -mercaptoethanol), centrifuged for 5 min at 1,200g to remove clumped particles and then at 8,000g for 10 min. The final pellets were dispersed in a few tenths ml of wash medium resulting in mitochondrial suspensions containing 20 to 30 mg protein/ml.

Similar procedures were followed in the isolation of mitochondria from Bartlett pears with the exceptions, based on prior experiments (25), that the PVP concentration was increased from 0.2 to 0.5% (w/v) and the pH during maceration was maintained within 6.7 to 7 by dropwise addition of 1 M KOH.

**Assay and Incubation.** Oxidative activity of mitochondria at 25 C was measured with a Clark-type polarographic electrode. The sequence of additions into the cuvette was as follows: 3 ml of reaction medium, mitochondrial suspension, and repeated additions of ADP until anaerobiosis. The reaction mixture consisted of 0.25 M sucrose, 66 mM potassium phosphate buffer (pH 7.2), 1 mM MgCl<sub>2</sub>, 3.3  $\mu$ M CoA, 33  $\mu$ M thiamine pyrophosphate, 0.1 mM NAD, 1 mg/ml BSA, 100  $\mu$ g/ml chloramphenicol, and 10 mM substrate. Unless otherwise indicated,  $\alpha$ -KG was supplied as substrate for both incubation and assay.

For incubation at 25 C, done according to Romani and Özelkök (24), 3 ml of reaction medium were placed in a 20-ml beaker

to which were added mitochondria (1 to 2 mg of protein) and 0.4  $\mu$ mole of ADP. The beaker was then covered with parafilm and placed in a water bath shaker set at 25 C and a shaking speed of 50 strokes/min. To measure mitochondrial activity, the contents of a beaker were swirled gently for about 30 sec to introduce additional  $O_2$ . The mixture was then transferred to the cuvette for polarographic assay.

Respiratory control ratios and ADP/O values were calculated according to the method of Estabrook (5). RCR represent the average of at least two state III-state IV cycles. Oxygen consumption was expressed as nmoles  $O_2 \text{ min}^{-1} \text{ mg mitochondrial protein}^{-1}$ . Protein was determined by the Lowry procedure as modified by Miller (16).

## RESULTS

**Avocados.** All substrates tested, with the exception of malate, resulted in the retention of RC, *i.e.* survival, by mitochondria from fruits at the peak and postclimacteric states (Fig. 1). However, survival of mitochondria isolated from fruit on the climacteric rise was relatively short regardless of the substrate supplied. The results of incubation in the absence of substrate (lower

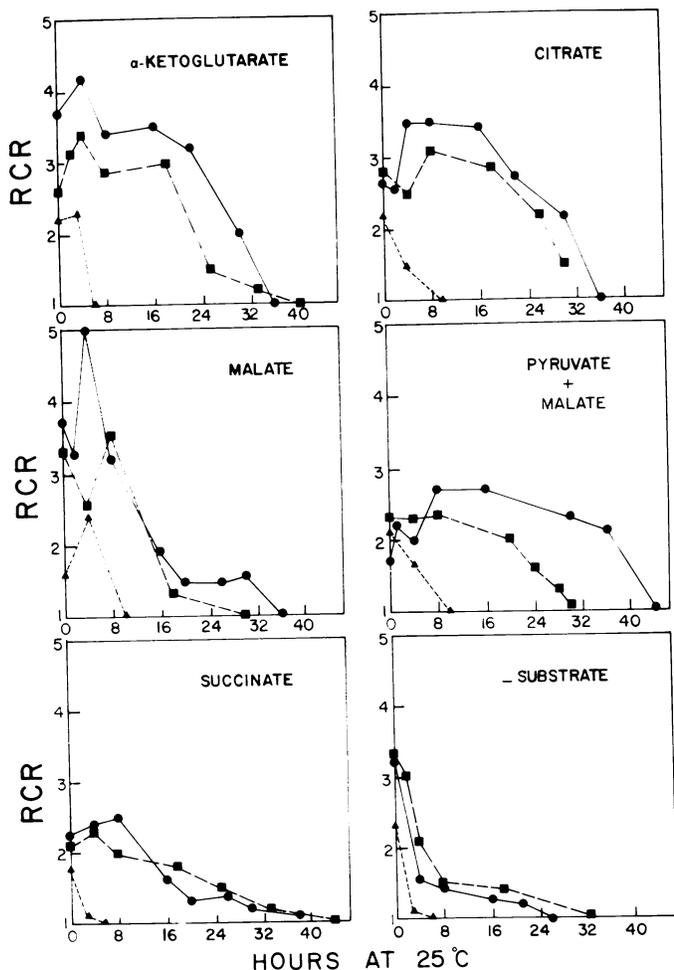


FIG. 1. Effect of substrate on the survival of avocado mitochondria isolated from fruits at three ripening stages. Isolations were made from fruit on the climacteric rise ( $\blacktriangle$ — $\blacktriangle$ ), at the climacteric peak ( $\blacksquare$ — $\blacksquare$ ), and at the postclimacteric ( $\bullet$ — $\bullet$ ) stage. In all experiments the medium contained 10 mM substrate except for pyruvate where 1 mM malate was also included as a sparker. In “- substrate”,  $\alpha$ -KG was added just prior to assay.

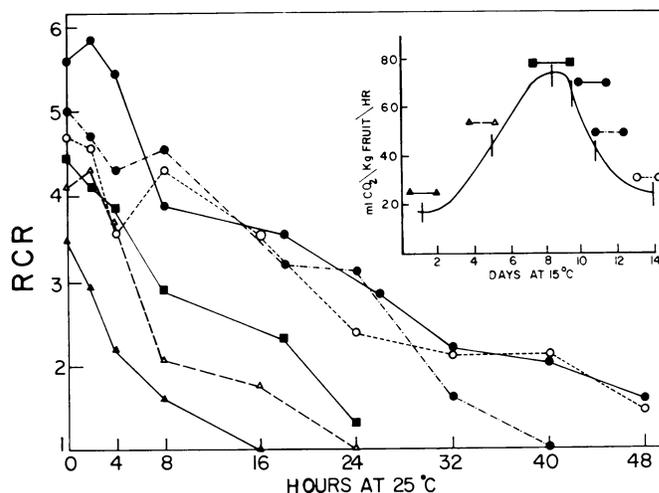


FIG. 2. Survival of avocado mitochondria as affected by physiological age of the fruit. Mitochondria were isolated from fruit at the climacteric minimum ( $\blacktriangle$ — $\blacktriangle$ ), climacteric rise ( $\triangle$ — $\triangle$ ), climacteric peak ( $\blacksquare$ — $\blacksquare$ ), early postclimacteric ( $\bullet$ — $\bullet$ ), mid-postclimacteric ( $\circ$ — $\circ$ ), and late postclimacteric ( $\square$ — $\square$ ) stages. Respiratory activity of the whole fruits and the times of the mitochondrial isolations are shown schematically in the inset.  $\alpha$ -Ketoglutarate was used as substrate.

right, Fig. 1) are most directly comparable with those where  $\alpha$ -KG was available to mitochondria throughout the incubation (upper left, Fig. 1). It is clear, as previously reported (23, 24), that active metabolism is required if the mitochondria are to retain RC *in vitro*.

To investigate the pattern of mitochondrial survival in relation to more definitive stages of the climacteric and postclimacteric, respiratory activity and physiological state were assessed for several individual fruits. Mitochondria were then isolated from fruits at six distinct climacteric states: climacteric minimum, climacteric rise, climacteric peak, and early, mid-, and late postclimacteric (inset, Fig. 2). The RCR of freshly isolated mitochondria increased progressively as the fruit advanced from the preclimacteric stage, through the climacteric peak, to the early postclimacteric stage (Fig. 2). The initial RCR decreased in the late postclimacteric phase. Although the ability of avocado mitochondria to survive *in vitro* appears to be correlated with the initial level of RC during the climacteric rise, the relationship does not hold for fruit that have passed the climacteric peak.

For comparison, the patterns of change in initial RCR, ADP/O, state III rates of  $\alpha$ -ketoglutarate oxidation, and survival of mitochondria isolated at six distinct climacteric stages are represented as a percentage of their respective values at the climacteric minimum (Fig. 3). A progressive increase in each of the above functions is apparent until the fruit reach a point just past the climacteric peak. These findings with respect to the first half of the climacteric phase are in agreement with those of Lance *et al.* (14) and with Biale's (2) contention that “the machinery for energy generation is in full operation throughout ripening.” However, RCR and oxidation rates decline as the mitochondria were obtained from progressively more senescent, postclimacteric fruit. These mid- and late postclimacteric changes did not markedly affect the capacity of mitochondria to survive. Similar patterns of age-related change were observed in other experiments with ripening Hass and Fuerte avocados.

**Bartlett Pears.** RCR from an experiment in which mitochondria were isolated at six points along the climacteric are shown in Figure 4. A comprehensive view of the patterns of change in oxidation rate, RC, and survival is given in Figure 5. In contrast to

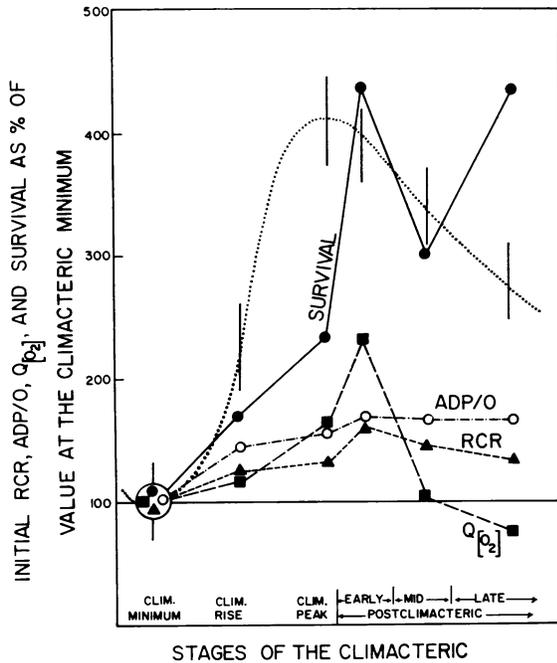


FIG. 3. Changes in initial RCR, ADP/O values, rate of  $\alpha$ -ketoglutarate oxidation (state III), and "survival" as percentage of values of the climacteric minimum. Vertical lines indicate the points along the climacteric curve (.....) when mitochondria were isolated from Fuerte avocados. Survival was based on the total number of hr at 25 C from the beginning of the incubation until loss of RC. The base functions at the climacteric minimum were RCR: 3.5, rate of oxidation (state III): 73 nmoles  $O_2$   $min^{-1}$   $mg$  protein $^{-1}$ , ADP/O: 1.6, and survival: 16 hr.

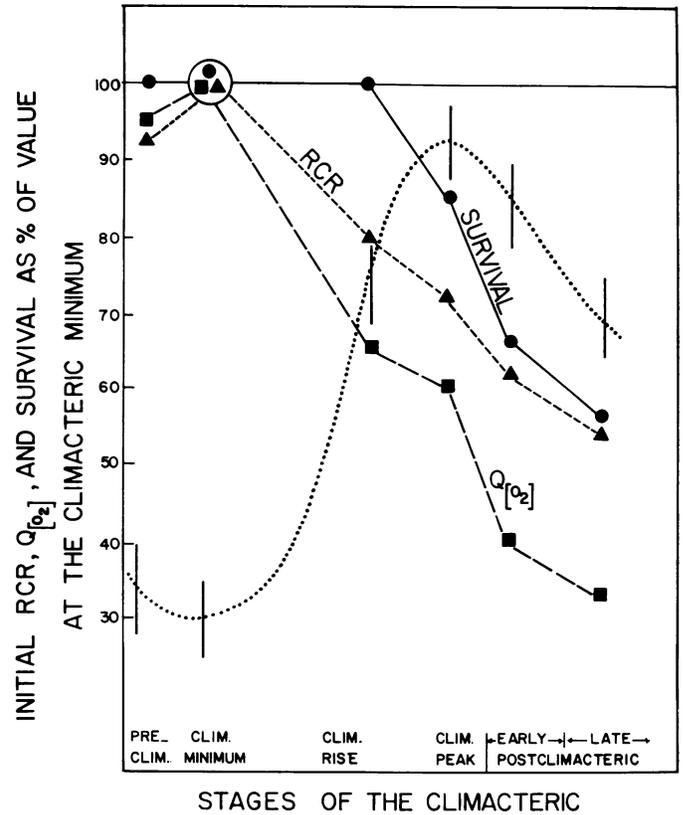


FIG. 5. Changes in initial RCR, rate of  $\alpha$ -ketoglutarate oxidation (state III), and "survival" as percentage of respective values at the climacteric minimum. The base functions at the climacteric minimum were RCR: 2, rate of oxidation (state III): 104 nmoles  $O_2$   $min^{-1}$   $mg$  protein $^{-1}$ , and survival: 48 hr.

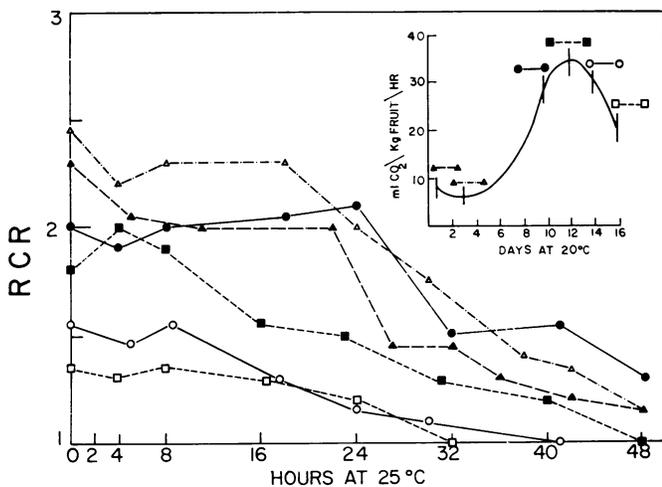


FIG. 4. Survival of pear mitochondria as affected by physiological age of the fruit. Mitochondria were isolated from fruit at the preclimacteric ( $\blacktriangle$ --- $\blacktriangle$ ), minimum ( $\triangle$ --- $\triangle$ ), climacteric rise ( $\bullet$ --- $\bullet$ ), climacteric peak ( $\blacksquare$ --- $\blacksquare$ ), early ( $\circ$ --- $\circ$ ), and late ( $\square$ --- $\square$ ) postclimacteric. Respiratory activity of pears at 20 C and approximate climacteric stages when mitochondria were isolated are shown in the inset.  $\alpha$ -Ketoglutarate was utilized as substrate.

the results obtained with avocados, pear mitochondria undergo an apparent functional decline with ripening and senescence of the parent fruit. However, the magnitude of the decline (40–50%) is considerably less of a change as compared to the 300 to 400% increase in the survival period of avocado mitochondria.

**Effect of Intraspecific Supernatant Fractions.** Any change in mitochondria isolated from tissues that are themselves undergoing a physiological transition raises the question of whether one is observing a change in the organelles *per se* or the influence of extra mitochondrial substances to which the organelles are exposed during isolation procedures. To help resolve this question, a portion of the mitochondria obtained from postclimacteric Fuerte avocados was incubated at 25 C in the usual manner. The remaining portion of mitochondria was divided into two equal parts. One part was gently dispersed in 30 ml of the supernatant fluid from which the mitochondria had previously been sedimented, and the other half was dispersed in the same amount of a preclimacteric avocado supernatant fluid. The mitochondria were allowed to incubate for 30 min at 0 C in the supernatant fractions and then recollected by centrifugation at 8,000g for 20 min, resuspended in a few tenths ml of wash medium (see "Materials and Methods"), assayed for RC, and then incubated at 25 C in the usual manner.

A deleterious effect of preclimacteric avocado supernatant fraction on the survival of postclimacteric mitochondria was clearly evident (Fig. 6). RC was lost after 16 hr of incubation, whereas it was retained in excess of 48 hr by the control mitochondria. Moreover, the pattern of decline in RC closely paralleled that of preclimacteric mitochondria (Fig. 6) whose supernatant fraction was utilized in the experiment. The slight positive response of the postclimacteric mitochondria to their own supernatant fluid indicates that the procedure itself had no harmful effects.

The experiment was repeated using postclimacteric avocados

of the Hass variety. As shown in Table I, both initial RCR and survival were reduced by exposure to the preclimacteric supernatant fraction. The lower RCR were clearly the result of reduced state III rates with very little change in state IV. ADP/O values also declined as a result of exposure to the preclimacteric supernatant fraction.

In experiments (not shown) in which preclimacteric mitochondria were exposed to a postclimacteric supernatant fraction, no enhancement of survival was observed. Even if benefits could have been imparted by the postclimacteric supernatant fraction, it is very likely they would have been overridden by unavoidable exposure (during isolation) of mitochondria to the preclimacteric supernatant fluid.

**Effect of Interspecific Supernatant Fractions.** The interaction of supernatant fractions and mitochondria from pears and avocados was tested by utilizing the procedures described above. Exposure of pear mitochondria to preclimacteric avocado supernatant fraction suppressed the initial RCR 30% and shortened survival

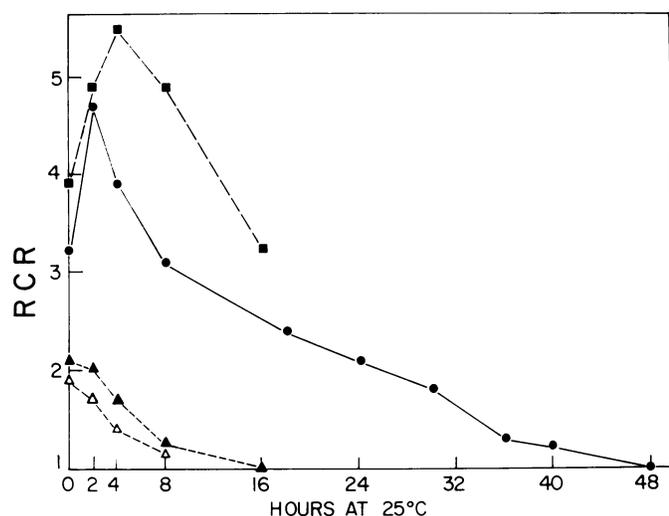


Fig. 6. Effect of preclimacteric avocado supernatant fraction on the survival of postclimacteric avocado mitochondria. Mitochondria isolated from soft, postclimacteric, Fuerte avocados (●—●) were exposed to preclimacteric (▲---▲) and postclimacteric (■---■) supernatant fractions prior to incubation as described in text; preclimacteric mitochondria sedimented from the supernatant fraction used in the experiment (△---△).

(Fig. 7). Additional data from the experiment (Table II) revealed that the improved RCR following incubation of pear mitochondria with postclimacteric avocado supernatant fraction were largely the result of decreased state IV rates, whereas treatment with the preclimacteric avocado supernatant fraction has its most marked effect on state III rates.

Under reciprocal conditions, wherein mitochondria from postclimacteric avocado fruits were exposed to supernatant fractions obtained from pears on the climacteric rise or at an advanced postclimacteric state, the supernatant fluid had no apparent effect. The decrease in survival capacity of pear mitochondria with advancement of ripening may thus be a true change in the organelles.

## DISCUSSION

Lance *et al.* (15) suggested that lack of cofactors, especially thiamine pyrophosphate, could limit the oxidation of  $\alpha$ -KG by mitochondria from preclimacteric avocado fruit. However, all cofactors, *i.e.*  $Mg^{2+}$ , BSA, NAD, thiamine pyrophosphate, and CoA shown to be required for maximal oxidation of Krebs cycle acids by avocado mitochondria isolated from all stages of ripe-

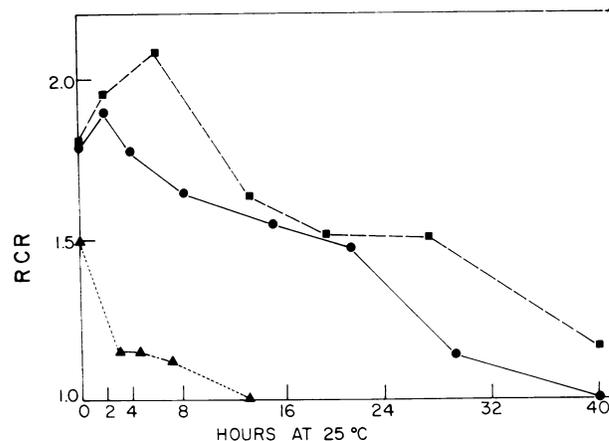


Fig. 7. Effect of pre- and postclimacteric avocado supernatant fractions on the survival of pear mitochondria *in vitro*. Untreated pear mitochondria (●—●), and similar mitochondria exposed to pre- (▲---▲) and postclimacteric (■---■) avocado supernatant fractions.

Table I. Effect of Prelimacteric Avocado Supernatant Fraction on Survival of Postclimacteric Avocado Mitochondria *In Vitro* at 25 C

Time at 25 C	Postclimacteric Mitochondria (control)			After Exposure to Prelimacteric Avocado Supernatant Fraction			After Exposure to Homologous Supernatant Fraction		
	Q <sub>O<sub>2</sub></sub> <sup>1</sup> (protein)	RCR	ADP/O	Q <sub>O<sub>2</sub></sub> (protein)	RCR	ADP/O	Q <sub>O<sub>2</sub></sub> (protein)	RCR	ADP/O
hr									
0	67 <sup>2</sup> (22) <sup>3</sup>	3.0	2.1	57 (21)	2.7	1.5	60 (20)	3.0	2.1
2	80 (27)	3.0	1.8	45 (26)	1.7	1.0	66 (24)	2.8	1.6
4	67 (21)	3.2	1.8	30 (20)	1.5	0.9	49 (21)	2.3	1.8
8	62 (28)	2.2	1.7	31 (22)	1.4	0.7	47 (23)	2.0	1.5
16	48 (24)	2.0	1.5	27 (24)	1.1	0.5	42 (24)	1.75	1.3
24	42 (26)	1.6	1.2				31 (23)	1.3	1.0
32	41 (29)	1.4	0.9				27 (23)	1.2	0.7
40	36 (29)	1.25	0.6				22 (20)	1.1	0.4
48	36 (30)	1.2	0.5				25 (25)	1.0	0.0

<sup>1</sup> Q<sub>O<sub>2</sub></sub> (protein): nmoles O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup>.

<sup>2</sup> State III rates.

<sup>3</sup> Numbers in parentheses: state IV rates.

ness (10, 14, 15, 27), were included in the incubation medium throughout these studies.

Biale and Young (3) suggested that an active ATPase could account for the low phosphorylative efficiency of mitochondria from preclimacteric avocados. Activation of a latent ATPase during incubation at 25 C could also explain the relatively short survival of preclimacteric avocado mitochondria were it not for the fact that the decrease in RC was, in most instances, due to reduced state III rates rather than increased state IV rates. This fact was clearly evident when pear mitochondria were utilized as a test source for avocado supernatant fractions (Table II).

Positive effects of a deproteinized supernatant fraction on  $O_2$  uptake by mitochondria from ripe avocados were reported by Romani and Biale (20) several years ago. Since then, procedures for isolating active, well coupled mitochondria from ripened avocados (10, 14, 27) were devised which obviated the "supernatant effect" and led to the understanding that mitochondria remained equally functional throughout the climacteric. It now appears that in terms of the capacity to maintain RC, exposure to preclimacteric avocado supernatant fluid is decidedly harmful. As judged from preliminary experiments (Table III), the harmful substance(s) in the preclimacteric supernatant fluid is neither a large, easily coagulated protein nor a small molecule readily removed by dialyses. In all likelihood the material is of vacuolar origin and hence not likely to exert an influence on mitochondrial metabolism in the intact cell. Nonetheless, identification of the harmful material present in preclimacteric supernatant fluid, as well as the nature of the beneficial effect of postclimacteric supernatant fraction (Fig. 7, Table III) should be pursued for clues to the requirements and susceptibilities of isolated mitochondria.

Since organelles are exposed to supernatant fluid in the process of their isolation, it has been virtually impossible to assess the true status of preclimacteric avocado mitochondria. This situation is not a singular experience. Damaging or masking effects from the unavoidable exposure of organelles to intracellular contents will frustrate any attempt to relate *in vitro* mitochondrial functions to the status of the organelles *in situ*. The damaging effects from intracellular contents can be discerned only if organelles not subjected to equivalent stress are available as a test system.

The difference in mitochondrial survival with the ripening of avocados and pears (Figs. 3 and 5) may be more apparent than real since the inferior state of preclimacteric avocado mitochondria is likely to have been experimentally induced by exposure to the harmful supernatant. The age-related decrease in survival capacity of pear mitochondria (Figs. 4 and 5), on the other hand, does correspond to a decrease in yield of intracellular particles

Table II. Survival of Pear Mitochondria *In Vitro* at 25 C as Affected by Exposure to Pre- or Postclimacteric Avocado Supernatant Fractions Prior to Incubation

Time at 25 C	Pear Mitochondria	After Exposure to Preclimacteric Avocado Supernatant Fraction	After Exposure to Postclimacteric Avocado Supernatant Fraction
hr	nmoles $O_2$ min <sup>-1</sup> mg protein <sup>-1</sup>		
0	71 <sup>1</sup> (39) <sup>2</sup>	41 (26)	62 (34)
2-3	74 (39)	34 (29)	54 (27)
4-6	66 (37)	30 (24)	54 (26)
13-15	53 (34)	23 (23)	47 (29)
20-21	35 (24)		37 (25)
29-30	35 (31)		33 (22)
39-40	23 (23)		21 (18)

<sup>1</sup> State III rates.

<sup>2</sup> State IV rates.

Table III. Response of Postclimacteric Avocado Mitochondria to Deproteinized or Dialyzed Supernatant Fraction

The mitochondria were treated by resuspension in fresh isolation medium or one of the various supernatant fractions for 30 min at 0 C, collected by centrifugation, and incubated at 25 C in reaction mixture as explained in the text. The "hr to loss of one-half initial RCR" bears an inverse relationship to the capacity for maintenance of RC and is approximately 50% of the survival period.

Treatment	None (control)	Fresh Isolation Medium	Preclimacteric Supernatant	Postclimacteric Supernatant	Deproteinized Preclimacteric Supernatant <sup>1</sup>	Deproteinized Postclimacteric Supernatant <sup>1</sup>	Dialyzed Preclimacteric Supernatant <sup>2</sup>	Dialyzed Postclimacteric Supernatant <sup>2</sup>
Initial RCR	3.3	3.5	2.6	3.8	2.5	4.6	2.8	4.2
Hr to loss of one-half initial RCR	35	40	20	40	20	41	20	34

<sup>1</sup> Deproteinized by subjecting to 100 C for 10 min, then chilled overnight at 0 C, and clarified by centrifugation at 10,000g for 10 min.

<sup>2</sup> Dialysis against 20 vol of isolation medium for 21 hr at 0 C.

(21) and in synthesis of mitochondrial protein (22) coincident with the climacteric rise in pear fruit. Moreover, Romani *et al.* (26) have demonstrated that the ability of pear cells to compensate for radiation damage to the mitochondrial system is lost or markedly impaired as fruits reach the climacteric peak. Since it is axiomatic that the ability to compensate for stress is essential for survival, the decline in survival of pear mitochondria in Figure 5 implies that intracellular compensatory processes are impaired with the cumulative stress of ripening and senescence.

The importance of utilizing, as criteria of the mitochondrial status, functions that are intrinsic to the undamaged organelle is evident from this study. Energy conservation and RC have most often served this purpose. However, the maintenance of RC (Figs. 3 and 5) may be even more diagnostic, for it demands that some of the energy be utilized in compensatory processes. The capacity to maintain vital functions *in vitro* emphasizes the quasi-independent nature of mitochondria, a characteristic which has led to the suggestion by Romani (19) that the respiratory climacteric may be simply a mitochondrial response to the degradative processes occurring in the cytoplasm of ripening fruit cells.

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