Cellular Senescence, Radiation Damage to Mitochondria, and the Compensatory Response in Ripening Pear Fruits

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Abstract. A compensatory response, viz. in vivo recovery from radiation damage to mitochondria, occurs in preclimacteric pear fruits (Pyrus communis L.) treated with ionizing radiation. The compensatory response is absent or markedly impaired in senescent fruits irradiated at or near the climacteric peak. Senescent cells failed to recover from harmful effects of radiation on: 1) mitochondrial yield, 2) in vivo incorporation of amino acids into mitochondrial protein, and 3) mitochondrial respiratory control and ADP/O. A diminished response to "split-dose" irradiation and a delayed rate of recovery confirmed the degeneracy and loss of compensatory power with cell age.

A loss of restorative activity, especially in mitochondria that supply the cell with essential energy, may underlie the more obvious signs of cumulative stress that accompany cellular senescence. Use of ionizing radiation as an investigative tool and the molecular implications of radiation damage, recovery, and cellular senescence are discussed.

It is generally accepted that the climacteric phase of ripening fruits is well suited for the study of cellular senescence (1). Since a surge in respiratory activity is the dominant, readily measurable phenomenon, mitochondria are implicated as the sites of causal events. This view was embodied in the early work of Millerd et al. (19) with the resulting postulate that uncoupling of oxidative phosphorylation permitted an increased respiratory rate. Along similar lines, Hulme (7) and Pearson and Robertson (21) attributed the rise in respiration to the availability of phosphate acceptor (ADP) resulting from increased protein synthesis and accelerated demands for energy. However, with specific reference to mitochondrial protein, data on the yield of particulate protein (27) and on the incorporation of amino acids into mitochondrial protein (28) indicate a decline in synthetic activities as fruit reach the climacteric peak.

Richmond and Biale (23), Sacher (37), and especially Young and Biale (44) have questioned the prevalence of "uncoupling" or "ADP control" in ripening fruit cells. Moreover, the work of Lance et al. (10), Hulme et al. (8, 9) and the experiments reported below, reveal that mitochondria remain functionally sound and "coupled" throughout the climacteric phase.

The persistence of active, phosphorylating mitochondria implies their continued essentiality to the senescent cells. More specifically, energy producing mitochondria may be vital to compensatory reactions essential for the survival of aging cells (38, 43). In this context, a change in the ability of mitochondria to compensate for stress, temporal or other forms, is a more critical age-dependent transition than changes in mitochondrial activity per se.

We have previously shown that massive, in vivo irradiation of preclimacteric pear fruits results in a loss and subsequent recovery of mitochondrial protein and an actual enhancement of mitochondrial activity (32). Similarly irradiated fruit cells also exhibit a suppression and recovery in the capacity to incorporate amino acids into mitochondrial protein (29). Utilizing respiratory control (RC) as an index to mitochondrial function, it was further demonstrated that RC is lost and then restored, in vivo, following massive irradiation of preclimacteric pear fruit (34).

Evidence has therefore been provided that preclimacteric pear fruit cells retain a capacity to repair radiation damage to mitochondria. At the same time, radiation stress also provided a tool with which to assess the level of compensatory activity as a function of cell age. Accordingly, with each radiobiological study experiments were also carried out utilizing pears at or near their climacteric peak. We present these age-comparative aspects in this paper and purport to demonstrate a marked decline in compensatory power with progressive senescence and the approach of the climacteric peak.

Preliminary reports of this work have appeared elsewhere (30, 35).

1 This investigation was supported by the United States Atomic Energy Commission, Contract AT (11-1)-34, Project No. 112, Report No. UCD34P112-32.
2 Preclimacteric and "climacteric minimum" will be used interchangeably as will "climacteric" and "climacteric peak."
Materials and Methods

Treatment of Fruit. ‘Bartlett’ and ‘Bosc’ pears (Pyrus communis L.) were obtained from local orchards and stored for several days at 0 to 2°C before use. With the exception of “split-dose” experiments for which fruits were taken directly from cold storage, all samples were first placed in respiration jars at 20°C and CO₂ production measured (3). At either their climacteric minimum or near their climacteric peak, pears were taken from the jars and irradiated with cobalt-60 gamma rays. A uniformity of absorbed dose within ±10% was assured by prior ferrous sulfate dosimetry (33). Dose rates over the period of these experiments ranged from 0.28 to 0.35 Mrad/hr. Temperature during irradiation ranged from 0°C to 5°C. After exposure the fruit were either kept chilled (0°C-5°C) for the isolation of mitochondria or returned to respiration jars at 20°C.

Split-Dose Irradiation. A normal irradiation treatment was given except that only a portion of the total dose was administered at 1 time. Between dose increments the fruits were returned to respiration jars at 20°C.

Isolation of Mitochondria. For determinations of mitochondrial yield, peeled, grated, and homogenized pear tissue was first lipolyphilized, and then reconstituted prior to the isolation of mitochondria. Details of the isolation procedure have been given elsewhere (27). “Yield” was defined as the amount of protein in the once-washed mitochondrial pellet, expressed as a percent of the protein, both mitochondrial and soluble, remaining in suspension after the first low speed centrifugation. Hence, anomalous results from differential cell-breakage were largely avoided.

Fresh tissue and added precautions were necessary to obtain coupled mitochondria from pears, or apples (34). Use of polyvinylpyrrolidone (PVP), as described by Hulme et al. (8) and carefully controlled pH during gentle maceration were prime essentials. Wiskich (42) also reported similar requirements for apple mitochondria. In brief, approximately 60 g of peeled, cold, pear tissue were gently cut and macerated into 180 ml of isolation medium consisting of 0.5 M sucrose, 0.05 M tris (pH 6.8), 0.006 M EDTA, cysteine (2 μmoles/gm tissue), 0.5% PVP (40,000 MW), and 1 mg/ml bovine serum albumin. The pH was monitored continually during maceration and kept between 6.8 and 7.0 with dropwise additions of KOH.

Centrifugations, first for 10 minutes at 1200 × g and then 15 minutes at 18,000 × g, were used to remove debris and to sediment the mitochondria. The centrifugations were repeated to obtain once-washed and once-clarified mitochondria.

Incorporation of Amino Acids. Procedures have been reported in detail elsewhere (29). Tissue slices were first incubated for 4 hours in a buffer solution containing 14C-leucine (0.03 μc pm l). Mitochondria were then isolated from the slices and the mitochondrial protein precipitated with trichloroacetic acid and resolubilized in base. After a second cycle of precipitation and solubilization in NH₄OH, the final protein solution was placed on lens paper and dried in preparation for liquid scintillation counting.

Assays. Utilization of O₂ by the mitochondria was measured with a polarographic oxygen electrode (34). Respiratory control (RC) was determined as described by Chance and Williams (2). To normalize the slight increase in RC known to occur with each successive addition of ADP (34) State 3 and State 4 rates were always calculated at the third (penultimate) addition of ADP. The final addition of ADP resulted in a renewed State 3, assuring a sufficiency of O₂ in the reaction mixture.

Proteins were assayed with a modification of the Lowry method (18).

Results

Respiration of Whole Fruit. With most fruits and vegetables irradiation causes a burst of respiratory activity (15,16). Pertinent to this study is our earlier observation that the magnitude of the respiratory rise is markedly reduced when fruit is irradiated at or near the climacteric peak (25,31).

Typical post-irradiation respiratory rates as affected by dose [0.25 and 1 million rad (Mrad) and climacteric state at time of irradiation, are shown in figure 1. The respiratory rates of pears irradiated at the climacteric minimum undergo increases of 300 to 400%. In contrast, similar doses applied at or near the climacteric peak elicited only a 20 to 40% increase. The respiratory rise caused by 1.0 Mrad is transient, leading rapidly into a suppressed activity. This transition from sustained to declining post-irradiation respiratory rates generally occurs in between 0.4 and 0.6 Mrad (25) and could be con-

Fig. 1. Respiratory activity of Bartlett pears irradiated at either the climacteric minimum or near the climacteric peak as indicated by the twiggie arrows. Unirradiated controls ( ), 0.25 Mrad dose ( , ), 1.0 Mrad dose ( ). Approximate stages of the climacteric are indicated at the top.
The unresponsiveness of older fruit cells in terms of mitochondrial yield correlates with the suppressed respiratory response (fig 1). For the climacteric fruit used in the yield determinations, the respiratory responses to 0.3 and 0.6 Mrad were not only minor but also indistinguishable.

**Incorporation of Amino Acids.** The influences of irradiation and cell age on mitochondrial yields, noted above, are substantiated by data on the rate of amino acid incorporation into mitochondrial protein. We have previously shown that there is 1) a marked decline in rate of incorporation as fruit reach their climacteric peak (28); 2) a suppression and subsequent recovery in incorporation rate following a 0.25 Mrad radiation dose to preclimacteric pears (29); and 3) a more drastic suppression with no recovery following a 1.0 Mrad dose to similar preclimacteric fruit (29). The question remained as to the effects of fruit age at time of irradiation. Data from a typical experiment examining the age-effect are shown in table I.

An approximately 50% inhibition caused by 0.25 Mrad was reversed within 24 hours in preclimacteric cells; there was no such reversal in pear cells near their climacteric peak. A 1.0 Mrad dose resulted in a greater and irreparable loss of activity regardless of the climacteric state.

**Recovery of Mitochondrial Functions.** The perturbations implied in the preceding experiments called for an assessment of their effects on mitochondrial activity. For this purpose RC, ADP/O, and Qo, (protein) were used as indices. A consistent pattern of suppression and recovery of these functional properties was noted following the irradiation of preclimacteric pears (34). However, as shown in figure 3, the performance of mitochondria from pears irradiated at the climacteric peak was quite different.

Each oxygen electrode trace (fig 3) was obtained with mitochondria from fruit given an identical, 1.0 Mrad radiation dose. Representative fruit samples were macerated and mitochondria isolated either immediately after exposure or after 24 hours at 20°C. Oxidative activity was suppressed and RC completely eliminated by the 1.0 Mrad dose regardless of climacteric stage. However, after 24 hour mitochondria

![Graph](image)

**Fig. 2.** Yield of mitochondrial protein from pear fruit tissue that had received 0.3 ( ), 0.6 ( ), 1.0 ( ) Mrad or 0.6 ( ) Mrad of ionizing radiation at the climacteric minimum (A) or near the climacteric peak (B). The yield of mitochondria from nonirradiated pears, ( ). Yield = particulate protein expressed as a percent of total protein remaining in the supernatant fraction after the first low-speed centrifugation. Each point in part A is the average of 6 separate isolations and in part B the average of 7 isolations. Methods for statistical analyses of the data were discussed elsewhere (32).

<table>
<thead>
<tr>
<th>Radiation dose</th>
<th>Radio-specific activity</th>
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</thead>
<tbody>
<tr>
<td>Mrad</td>
<td>Preclimacteric</td>
</tr>
<tr>
<td></td>
<td>1 hr¹</td>
</tr>
<tr>
<td>0</td>
<td>55.7(±1.4)</td>
</tr>
<tr>
<td>0.25</td>
<td>24.8(±0.4)</td>
</tr>
<tr>
<td>1.0</td>
<td>6.0(±1.8)</td>
</tr>
</tbody>
</table>

¹ Approximate time interval between irradiation of the pear fruit and preparation of tissue slices. Values are the average (cpm/mg protein) in 2 separate incubations of pear tissue slices.

² Pears were irradiated approximately 24 hours before they would have reached their climacteric peak.

Table I. Incorporation of ¹⁴C-Leucine into Mitochondrial Protein as a Function of Radiation Dose, Stage of Cell Senescence, and Time Interval Between Radiation Exposure and Introduction of Labeled Amino Acids
in preclimacteric fruit had recovered RC and oxidative activity to approximately control levels (legend, fig 3). There was no sign of recovery by mitochondria in climacteric fruit.

Data from a more comprehensive experiment are given in table II. In preclimacteric pears radiation damage was not only countered but over-compensated, resulting in an RC overshoot, e.g. RC values of 7.5 and 10 compared to a maximum of 3.9 for the non-irradiated controls. The radiobiological implications of the overshoot and repair kinetics are discussed elsewhere (36). For the purpose of assessing the effects of senescence it is significant that there was no overshoot in RC by mitochondria in climacteric peak fruit. Even after 0.25 Mrad RC was never restored to control levels.

Table III contains the ADP/O and Q_{H2} (protein) values obtained concomitantly with the RC data. With only 1 exception, the ADP/O values following 0.25 Mrad indicate a reasonably efficient conservation of energy from the oxidation of &alpha;-ketoglutarate, despite radiation injury or climacteric state. A 1.0-Mrad dose, however, results in a marked lowering of the ADP/O, and a subsequent recovery only in preclimacteric cells.

$Q_{H2}$ (protein) values establish the general level of metabolic activity. However, co-precipitation of variable amounts of inactive protein during centrifugation of the mitochondria render these values unreliable as indicators of mitochondrial condition. This is reflected by the variability in $Q_{H2}$ values (table III). Nonetheless, it is significant that some oxidative activity persists after 1.0 Mrad, with a sign of considerable post-irradiation recovery by mitochondria in preclimacteric fruit.

Response to Split-Doses. Classical "split dose" techniques have been used to demonstrate the presence of mitochondrial repair, or de novo synthesis, in irradiated fruit cells (36). The basic assumption in "split-dose" experiments is that repair, or reconstitution, must have occurred in the interval between dose increments when the overall radiation tolerance is increased (4). Data from a set of experiments comparing the response to split-doses by mitochondria in preclimacteric and climacteric pears are summarized in figure 4. A 1.5 Mrad dose was required to completely obliterate RC ($Q_{H2}$ state 3 : $Q_{H2}$ state 4 = 1) in mitochondria from this lot of preclimacteric pears (fig 4, upper graph). However, when administered in 3 doses of 0.5 Mrad each, with 24 hours intervening between increments, the same total, 1.5-Mrad, dose resulted in only a 50% decrease in RC. These results are in marked contrast with the performance of mitochondria in similar pears irradiated at their climacteric peak (fig 4, lower graph). Not only was the radiation tolerance lowered and RC completely lost after only 0.75 Mrad, but partial repair could be demonstrated only if the second dose was limited to 0.25 Mrad.
Table III.  ADP/O and Qo₂ (Protein) Values Obtained During the Oxidation of α-Ketoglutarate by Mitochondria Isolated at Different Time Intervals After Irradiation of Whole Pear Fruit

<table>
<thead>
<tr>
<th>Irradiation</th>
<th>Climacteric minimum</th>
<th>Climacteric peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>Days</td>
<td>ADP/O²</td>
<td>Qo₂ (Protein)²</td>
</tr>
<tr>
<td>0</td>
<td>2.7</td>
<td>2.8</td>
</tr>
<tr>
<td>1</td>
<td>3.1</td>
<td>4.2</td>
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<tr>
<td>2</td>
<td>2.7</td>
<td>2.8</td>
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<tr>
<td>4</td>
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</tr>
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<td>2.7</td>
<td>(…)</td>
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<tr>
<td>0</td>
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<tr>
<td>4</td>
<td>43</td>
<td>(…)</td>
</tr>
<tr>
<td>5-6</td>
<td>97</td>
<td>(…)</td>
</tr>
</tbody>
</table>

1 Fruit were held at 20° for the indicated period of time, chilled for approximately 2 hours, and the mitochondria isolated.
2 Conditions of assay for ADP/O and Qo₂ (protein) given in legend of figure 3.
3 Low ADP/O values are not discernible in the absence of RC.
4 Instances where fruit were discarded because of excessive radiation damage and/or decay.

Discussion

Use of Ionizing Radiation. Since ionizing radiation was utilized as an investigative tool, it is perhaps well to consider certain characteristics of radiation damage before discussing the experimental results. Setlow and Pollard (39) have defined important biophysical aspects of radiation damage and Romani (26) has reviewed these aspects in relation to the high dose irradiation of plants and plant parts. The following points are especially relevant to the present experiments. 1) Damage from ionizing radiation is all pervasive and uniformly distributed, e.g. assuming a mitochondrial volume of 4 μ³, at an absorbed dose of 0.1 Mrad each organelle is the site of approximately 7 × 10⁶ ionizing events. 2) Damage is at the molecular level and, if not excessive, amenable to biochemical (molecular) repair mechanisms. 3) The amount of cellular damage (radiation dose) can be readily quantitated to incipient, reparable, or irreparable levels.

Thus, as opposed to age-stress, ionizing radiation has defined temporal and spatial parameters. Moreover, it can be administered in fixed increments.

Of added importance to this study is the effect of radiation in ameliorating the influences of ripening changes on homogenization procedures and the isolation of organelles. As discussed by Hanson (6) and by Romani et al. (27), analyses of temporal, quantitative, or functional changes in mitochondria are subject to the vagaries of isolation techniques. The problem is rendered even more difficult by the physical and chemical changes that occur in ripening fruits. Radiation is effective in 2 ways. First, obvious physical and textural changes in the tissues result from the doses used. This imposed textural change will have its own effect on maceration procedures, diluting and somewhat normalizing any impact of ripening changes. Second, and more

Fig. 4. Comparing the response to "split doses" by mitochondria in preclimacteric pear cells (upper graph) and in climacteric pear cells (lower graph). Respiratory control (RC) values are for mitochondria isolated from: 1) nonirradiated pears, 2) pears irradiated in single exposures, or 3) pears irradiated in Mrad increments as indicated at the top of each bar. On the abscissa, line a = time between doses, line b = cumulative time from first exposure.
important, radiation damage elicits a cellular response (mitochondrial repair or de novo synthesis) that takes place in vivo preceding and thus largely obviating isolation artifacts.

**Stress and the Compensatory Reactions of Senescent Cells.** As postulated by Oldfield (20) every stress affecting the cell induces repair processes which tend to restore the normal state. Samis (38) outlines the role of compensatory repair processes in counteracting age-stress. Data demonstrating increased nucleic acid and protein metabolism in the liver of aging rats are presented by Wulff et al. (43) as signs of accelerated synthetic activity compensating for age-stress. However, in the same paper, it is acknowledged that evidence also exists in support of a diametrically opposed relationship between aging and synthetic reactions. As noted in the introduction to this paper, a similar divergency exists regarding the question of protein synthesis in senescent fruit cells.

Our experiments emphasize the loss in capacity of senescent pear cells to repair, or in some way compensate for radiation damage to mitochondria. This phenomenon was evidenced by several criteria including 1) the overall respiratory response of the cells, 2) quantitative changes in mitochondria, 3) rate of amino acid incorporation into mitochondrial protein, 4) time course of the mitochondrial recovery of RC, and 5) recovery following a split radiation dose. In all cases the climacteric respiratory pattern served in the selection of the 2 “age-states:” climacteric minimum and climacteric peak. Clearly, at some point during this time-span pear fruit cells lose their capacity to compensate for radiation damage to mitochondria.

The existence of a fundamental transition in moribund cells from adequate to inadequate compensatory mechanisms could explain some of the conflicting reports cited by Wulff (43) and in the introduction to this paper. As a case in point, the decrease in mitochondrial yield as pear fruit reach the climacteric peak was, as reported (27), preceded by a slight increase in yield during the first portion of the climacteric rise. Similarly, Richmond and Biale (23) reported a rise in protein synthesis during the early phases of the climacteric prior to a decline in synthesis as fruit reach the climacteric peak.

**Cellular Senescence and the Respiratory Climacteric.** The age-dependent transitions noted above have been substantiated in repeated experiments with pear fruit obtained from different regions, and in the case of mitochondrial functions, with 2 pear varieties, Bartlett and Bosc. While it is accepted that the climacteric respiratory pattern is coincident with a senescent phase, it is questionable that the respiratory pattern is an absolute guide to physiological age. For instance, the production of volatiles during the respiratory climacteric in peaches is markedly altered by the stage of maturity at harvest (14). Cultural conditions during growth and the post-harvest treatment of fruit may well affect the subsequent cellular response to age (ripening), or other forms of stress. It should not be surprising, therefore, if age-related cellular functions reflect some variability in vivo a vis the climacteric. We have noted a variability in the radiation tolerance of mitochondria in pears that were, presumably, at similar preclimacteric stages: for instance, a loss of RC at 1.0 Mrad in one lot of fruit (table 11) and at 1.5 Mrad (fig 4) in another. This observation does not detract from the age-related loss of compensatory activity which is normalized within a given experiment and sample of fruit. It does suggest, however, that radiation stress may prove useful in unmasking transitions in other cellular processes that precede and perhaps control the onset of the climacteric phase.

**Molecular Aspects.** In another study attention was called to the overshoot in compensatory activity resulting in a 2 to 3 fold higher RC for the “recovered” mitochondria as opposed to organelles from unirradiated tissues (36). The occurrence of this overshoot, in concert with increased cellular respiratory activity, suggests that recovery of RC is mediated by energy requiring processes leading to the repair of damaged sites or de novo synthesis of mitochondria to replace the injured organelles. Van Stevenick and Lackman (41) also observed a loss and subsequent reconstitution of mitochondria following slicing of storage tissues, while Lee and Chasson (13) noted an increase in mitochondrial material with “aging” of potato discs. With regard to both studies it is important to distinguish between localized mechanical injury as occurs in the preparation of tissue slices and all-pervasive, molecular damage from radiation. A distinction should also be made between “aging” of tissue slices in vitro as opposed to normal physiological senescence and death.

It has been proposed that cellular senescence is the final phase of genetic expression (5,17,40) and hence, subject to the control mechanisms implicit in current theories of “molecular biology.” To the extent that compensation of radiation damage involves protein synthesis it too is subject to the same or similar control mechanisms. The interrelationship between protein synthesis and radiation repair is reinforced by the observation that almost complete inactivation of 70 and 80S ribosomes (12,22) occurs in the same dose range (0.6-1.2 Mrad) that results in irreparable injury to pear mitochondria. Pear ribosomes are 80S (11), and moreover, a rapid decline in their synthesis (Ku and Romani, unpublished observation) has been found coincident with the final portion of the climacteric rise. Richmond and Biale (24) have also reported a marked decline in RNA synthesis as avocados reach the climacteric peak.

**Concluding Remarks.** Our findings indicate that a critical intracellular transition occurs during the latter portion of the climacteric rise in senescent fruit cells leading to a degeneracy in compensatory power. Because of the apparent involvement of pro-
tein synthesis, the transition may stem from a degeneracy in RNA synthesis, as suggested by Richmond and Biale (24), or from a programmed change at any one of the many control points manifest in current molecular theory of protein and RNA synthesis.

As it applies to mitochondria, a degeneracy in compensatory activity may be especially critical if the organelles are to supply the senescent cell with energy to counter age-stress.

Literature Cited


