Plant Physiol. (1979) 63, 814–817
0032-0889/79/63/0814/04/$00.50/0

Senescence of Pear Fruit Cells Cultured in a Continuously Renewed, Auxin-deprived Medium

Received for publication November 7, 1978 and in revised form February 2, 1979

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ABSTRACT

Auxins and cytokinins support cell division in tissue and cell cultures. In cytokinin-independent pear (*Pyrus communis*) cells, omission of 2,4-dichlorophenoxyacetic acid (2,4-D) from the medium for two successive transfers leads to rapid cell lysis, unless the osmolarity is raised to 0.4 molar with mannitol. Use of this system (nutrients plus mannitol minus 2,4-D) for the study of cell senescence was explored both in batch culture and in a system designed to permit medium renewal without withdrawal of live cells.

In both systems, an initial period (1–6 days) of limited increase in cell number is characterized by a continuous decrease in the respiratory activity and in protein and RNA synthesis to very low basal rates. In batch culture, cell death occurs after 13 to 15 days with little or no change in metabolic activity, or in protein and RNA synthesis. With renewal of cell medium, death is slightly delayed and is preceded by a burst in RNA synthesis followed by a notable increase in protein synthesis. Cycloheximide inhibition of protein synthesis is transient and its effect on cell longevity variable. Nonetheless, in all instances cell death is preceded by a burst in protein synthesis. Actinomycin D (1.6 micromolar) did not significantly affect protein synthesis but delayed RNA synthesis and cell death. The possible roles of auxin, osmoticum, and macromolecular synthesis in cellular senescence and death are discussed.

Strong auxins, such as 2,4-D, are required to support cell division in tissue and cell cultures of apple or pear fruits (25, 28). Under such conditions the cells, whether initiated from young or mature fruits, do not exhibit senescence (27). Moreover, no symptoms of senescence have been obtained in callus cultures upon treatment with ethylene or its precursors (24). Auxins appear to be a major factor in the avoidance of senescence, and the lack of an auxin in the medium leads to a rapid lysis of cultured pear cells (27). The same phenomenon has been observed in *Acer* cultures (9), but not in carrot cells (32). Of particular interest was the observation by Codron *et al.* (5) that increasing the osmolality of the medium by means of mannitol prevents rapid cell death in the absence of exogenous auxin thus providing a means of achieving a delayed senescence.

Some signs of cellular senescence have been observed after nutritional shifts in tobacco cells (2) and during the stationary phase of carrot cells (26). In neither of these studies was the senescent phase well marked nor was the hormonal balance of the experimental system considered.

In this paper we report on the effects of auxin deprivation on macromolecular synthesis and cell death in suspension cultures of Passe Crassane pear cells in batch condition, and in a newly devised system for continuous renewal of the medium. A preliminary report of the work has appeared (29).

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MATERIALS AND METHODS

Cell Cultures. The strain of cells used in these experiments was established in 1972 from young Passe Crassane pear fruit (*Pyrus communis* L.).

The standard medium used for cell growth consisted of the mineral nutrients of Murashige and Skoog (23), the organic nutrients of Nitsch *et al.* (25), as slightly modified by Pech *et al.* (28), and 2,4-D (4.5×10^{-6} M). Cytokinins were omitted after 2 years of culture without any effect on growth intensity. The cultures were grown under incandescent light at 27 C on a platform shaker (100 rpm) and subcultured every 7 days using about 10% inoculum. The medium with reduced level of nutrients developed by Codron *et al.* (5) for maintenance and eventual senescence of cells under auxin deprivation consisted of one-fourth concentration of the mineral and organic nutrients present in the standard medium plus either 0.4 M mannitol or 0.37 M mannitol + 0.03 M sucrose. This has been termed the aging medium.

Before transferring into aging medium the cells were grown for 12 days in the standard medium in absence of 2,4-D at an initial cell density higher than 5×10^5 cells/ml. Prior to transfer, the cells were allowed to settle and were then washed twice with aging medium.

Culture in Batch Conditions. Three-liter flasks containing 0.8 liter of aging medium were inoculated with 0.2 liter of cells prepared as described above. The methods for aeration, shaking, and sampling were the same as those for the continuous system described in Figure 1.

Culture with Continuously Renewed Medium. The objective was to renew the medium continuously without removing live cells. A schematic diagram of the system is shown in Figure 1.

A 2.8-liter Fernback flask with wide surface area containing 2 liters of medium was used. Withdrawal of the culture liquid was via a cell separator, a section of tubing sufficiently large (17 × 150 mm) to reduce greatly the speed of the outflow and thus allow live cells to settle and reenter the flask. Filters to separate cells and medium were tried, but proved to be ineffective. Sampling, or addition of cofactors, inhibitors, etc., was done by means of a sterile needle and syringe through the side arm. Silicone tubing (4 mm o.d., 2 mm i.d.) was used throughout. Air at 8 p.s.i. was sterilized by passage through a glass wool filter and monitored by flow meters. Four similar systems were operated simultaneously on the same rotary shaker adjusted to 100 rpm.

Protein, DNA and RNA Synthesis. The assay procedure of Ferrari and Widholm (10) was used with the following modifications. An aliquot of cell suspension was first diluted with fresh aging medium to a final concentration of 1×10^5 to 2×10^5 cells/ml. To 5-ml aliquots of the diluted cells were added either 0.1 μCi of [U-^{14}C]leucine (302 μCi/mmol) or 0.2 μCi of [U-^{14}C]uridine (462 mCi/mmol) and then incubated for 2 h with shaking at 25 C.

Incorporation rates were a linear function of time and cell concentration over this period. Subsequent collection and washing procedures utilizing 2.4-cm glass fiber discs and a methanol-chloroform-water (12:5:3, v/v/v) mixture were unchanged. It was verified that the macromolecular synthesis measured by this method was quantitatively similar to that determined with a more rigorous but time-consuming trichloroacetic acid precipitation (7).

Respiration Measurements. CO_2 present in the effluent air was measured by the colorimetric method of Claypool and Keefer (3).

Estimation of Dead Cells. Dead cells were selectively stained by adding Evan's blue (0.25%, w/v) to the medium (15) and estimated after 10 min by counting under a light microscope. Four duplicate counts were made each time.

Ethylene. Ethylene production was measured by gas chromatography by taking a 2-ml sample of head space gas from 25-ml sealed flasks containing 10 ml of cell suspension shaken at 25 C for 2 h.

RESULTS

The response of pear cells to auxin deprivation and senescence in suspension culture was examined in the aging medium after a previous culture of 12 days in normal medium less 2,4-D.

Cellular Senescence in Batch Culture. In the presence of 0.4 M mannitol or 0.37 M mannitol + 0.03 M sucrose an increase in cell number was observed during the first 6 to 8 days of incubation (Fig. 2A). During this period respiration (Fig. 2B), RNA synthesis

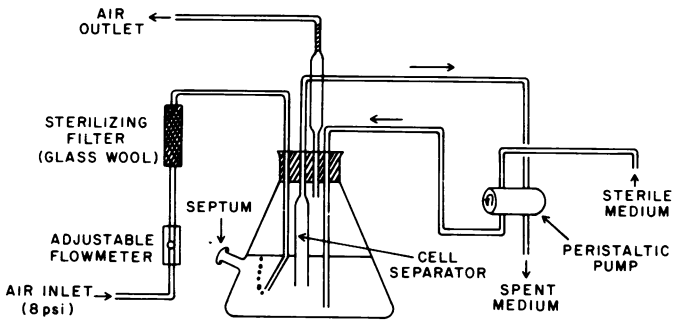

FIG. 1. System for cell culture which permits continual renewal of medium without removal of cells.

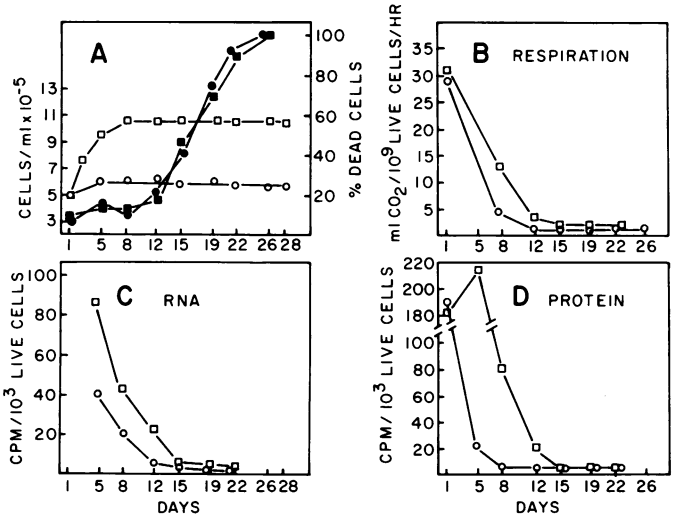

FIG. 2. Cell number and per cent dead cells (A), respiration rate (B), incorporation of [^{14}C]uridine (C), and incorporation of [^{14}C]leucine (D), by auxin-deprived pear fruit cells cultured in batch conditions, with minimal media including either 0.4 M mannitol (○) or 0.37 M mannitol + 0.03 M sucrose (□). Closed symbols (●, ■) represent per cent dead cells. Air flow rate: 20 ml min^{-1}. Each point represents mean of four samples, two each from duplicate cultures.

Table I. *Characteristics of Four Different Experiments Conducted with Continuous Renewal of Medium*

Expt. No.	Medium Flow Rate	Air Flow Rate	Increase in Cells Day 0 to Day 6	Day When Cell Death Begins	Increase in Synthesis Preceding Cell Death	
					Protein	RNA
	ml/day	*ml/min*	%		%	
1	268	40	30.5	16	122	
2	244	20	20.6	19	153	
3	254	30	12.5	17	300	540
4	284	30	17.0	17	184	386

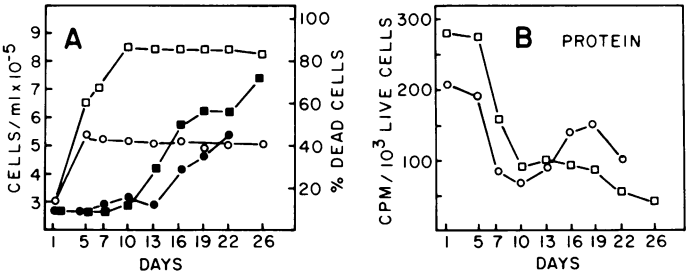

FIG. 3. Cell number and per cent dead cells (A) and [^{14}C]leucine incorporation (B) in auxin-deprived pear fruit cells cultured with continuous renewal of minimal medium containing either 0.4 M mannitol (○) or 0.37 M mannitol + 0.03 M sucrose (□). Closed symbols (●, ■) represent per cent dead cells. Medium flow rate: 280 ml day^{-1}. Air flow rate: 40 ml min^{-1}. Each point represents mean of four samples, two each from duplicate cultures.

(Fig. 2C), and protein synthesis (Fig. 2D) decreased very rapidly. Incorporation of [^{14}C]thymidine (not shown) was found only during the period of cell division. Ethylene production was initially very low (about 0.6 μl/h·10^{10} cells) and decreased steadily to a nondetectable level by day 12.

The somewhat more prolonged and higher rate of cell division in the presence of 0.03 M sucrose was accompanied by comparable delays in the decline of respiratory activity and in rates of RNA, and protein synthesis (Fig. 2). However, with or without sucrose, cell death occurred after the same time interval (Fig. 2A).

Since lack of metabolizable carbohydrate did not appear to be a determining factor in senescence and death, it was assumed that cell lysis could be due either to limiting nutrient(s) or accumulation of toxic compounds in the medium. For this reason, a system in which the medium could be continuously renewed was devised (Fig. 1). Characteristic flow and renewal rates of such systems are given in Table I.

Cellular Senescence in Continuous System. The prolongation of cell division in the presence of sucrose was roughly similar in the continuous (Fig. 3A) and batch systems (Fig. 2A). However, with continuous medium renewal and in the absence of sucrose, cell death was delayed by several days (Fig. 3A). A clear pattern of increase in protein synthesis was observed beginning at about the 10th day (Fig. 3B). The increase in protein synthesis was reproducible although it varied between about 120% to 300% in four different experiments (Table I). A notable burst in RNA synthesis (Table I) preceded protein synthesis (Fig. 4C, control).

Effects of Cycloheximide and Actinomycin D. Based on the observed sequence of events it seemed conceivable that macromolecular synthesis is directly related to the final period of senescence and cell death. To test this hypothesis, cycloheximide (1 μg/ml or 3.5 μM) and actinomycin D (2 μg/ml or 1.6 μM) were added to separate flasks and corresponding media reservoirs. The additions were made after 6 days of incubation to avoid an effect of the inhibitors on cell division (19).

In the presence of cycloheximide, protein and RNA synthesis was inhibited to a large extent (Fig. 4, C and D), but not completely. Partial recovery occurred after a few days. Compared to the control cells, respiratory activity in the presence of cyclohexi-

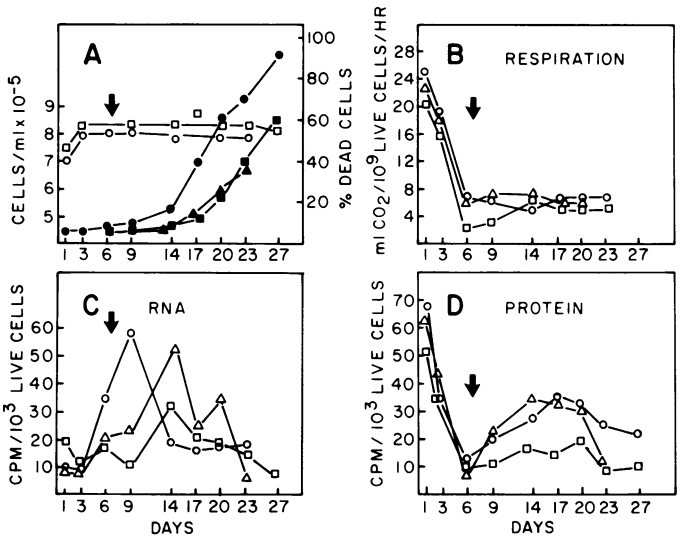

FIG. 4. Cell number and per cent dead cells (A), respiration rate (B), incorporation of [^{14}C]uridine (C), and incorporation of [^{14}C]leucine (D) in auxin-deprived pear fruit cells cultured with continuous renewal of minimal medium containing 0.40 M mannitol and macro and micro elements but no sucrose. Air flow rate: 30 ml min^{-1}. Medium flow rate: 290 ml day^{-1}. Cycloheximide (3.5 μM) or actinomycin D (1.6 μM) was added on the 6th day (arrows). Control (○), + cycloheximide (□), + actinomycin D (△). Closed symbols (●, ■, ▲) represent per cent dead cells.

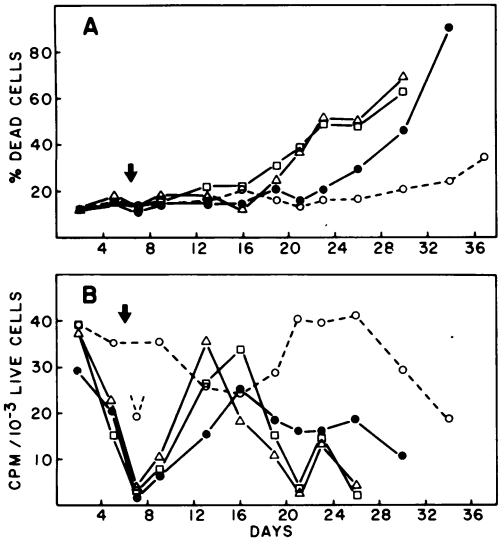

FIG. 5. Cell death (A) and incorporation of [^{14}C]leucine (B) in auxin-deprived pear fruit cells. Incubation conditions as described in legend of Figure 4. Control (○---○), 0.7 μM (●——●), 1.75 μM (△——△), or 3.5 μM (□——□) cycloheximide added on day 6 (arrow). CPM datum for control, day 7 is in question.

mide declined more rapidly and to lower rates, but then returned to the control level in a few days (Fig. 4B).

Actinomycin D significantly inhibited the rate of RNA synthesis for a few days, but it also did not have a permanent effect since a peak of RNA synthesis occurred 5 days later with nearly the same intensity as in the control (Fig. 4C). Protein synthesis and respiration were not affected by actinomycin D. In the preceding series of four separate experiments the death of control cells was observed to begin between day 16 and day 19 (Table I) and cycloheximide delayed death by 4 to 6 days. A few months later a new series of studies was begun. In three separate experiments, the control cells now survived for about 4 weeks and cycloheximide treatment caused an earlier death beginning on about day 16. Suspecting that a new batch of cycloheximide was more potent (less degraded!) lower concentrations of 0.7 and 1.75 μM (0.2 and 0.5 μg/ml) were also tested. As seen in Figure 5, the lowest concentration resulted in intermediate survival times confirming that in this population of Passe Crassane cells cycloheximide hastened cell death. The change in normal survival time and death response to cycloheximide is unexplained and may be one of the vagaries encountered with serially cultured cells. However, it is pertinent to observe that in every instance, with or without cycloheximide, cell death is preceded by a burst in protein syntheses (Fig. 5).

DISCUSSION

The strain of pear fruit cells used in these experiments was cytokinin autotrophic. But it is probable that as for tobacco callus (8), a minimum level of auxin was required to support cytokinin synthesis. Hence, auxin deprivation can also be considered cytokinin deprivation, and the system used in this study should prove useful for following the separate or combined effects of exogenous auxins and cytokinins on nondividing, hormone-starved cells.

King (18) noted that after switching chemostatic cultures to a minus 2,4-D medium there was a preferential retention of 2,4-D by the cells so that intracellular concentrations were still roughly 15% of the initial value after 20 days (Fig. 3 in ref. 18). The confinement of cell division to a few days after transfer to the aging medium (Figs. 2A, 3A, and 4A) may, as reasoned by Leguay and Guern (20), result from the gradual decrease of intracellular 2,4-D to a point below the threshold value required for division.

The possible mode of action of osmoticum in maintaining cellular integrity in the absence of exogenous hormones has been previously discussed by Codron *et al.* (5). Reduction in solute leakage and maintenance of intracellular 2,4-D are the most plausible explanations. However, the ability of 2,4-D at 1 mg/l to induce division of auxin-deprived cells in the normal medium but not in aging medium containing 0.4 M mannitol (unpublished results) indicates that the osmoticum may also increase the threshold value of 2,4-D required for cell division. Analogous effects of mannitol, but at concentrations above 0.5 molal, are implicit in experiments by Kimball *et al.* (17) with soybean tissue cultures. Marrè *et al.* (22) also observed that increasing osmolality had no effect on an auxin-stimulated pH decrease in pea internode segments but did suppress cell enlargement.

A decrease in rate of macromolecular synthesis after transfer of tobacco protoplasts to a higher osmoticum was observed by Premecz *et al.* (30). Nevertheless, synthesis continued at a linear rate for several hours. Its subsequent decline was attributed to exhaustion of hormones, an effect previously noted by Zelcer and Galun (33). Our observation that 2,4-D (1 mg/l) added to pear cells after 6 days in the aging medium fails to induce cell division and protein synthesis is further indication that cytokinin synthesis might be suppressed. This hypothesis is supported by the observations of Maab and Klambt (21) that cytokinins, but not auxins, stimulate protein synthesis in hormone-deprived tobacco cells whose RNA synthesis was blocked by actinomycin D. Cytokinins also induce polyribosome formation in suspension cultures of *Glycine max* (12).

Cell death in batch culture is not accompanied by an increase in macromolecular synthesis (Fig. 2), whereas with renewal of medium cell death is not only delayed but is preceded by a notable burst of RNA and protein synthesis. This difference between the closed and the open system could be explained by a difference either in the supply of nutrient or in the accumulation of toxic compounds. The fact that merely increasing the assimilable carbon source or minerals (data not shown) has no effect on macromolecular synthesis or cell death would make the second hypothesis more likely.

Enhanced protein and RNA synthesis in the continuous system could be interpreted as the synthesis of new enzymes involved in

senescence and cell death. This view is supported by the consistent temporal relationship between macromolecular synthesis and cell death, in spite of differences in survival times and in response to cycloheximide. In this context, it would be interesting to test the effects of polyamines which have been shown to delay senescence of oat leaf protoplasts (1) by controlling ribonuclease activity (16). Secondary effects must also be considered. For instance, we have observed that cycloheximide, but not actinomycin D, induces cyanide-insensitive respiration (under further investigation).

In this study we did not examine inhibitor effects on uptake, precursor synthesis, and pool size. Other studies with cultured plant cells provide evidence that the primary action of cycloheximide and actinomycin D is on macromolecular synthesis (11, 13). Incomplete inhibition by cycloheximide and recovery of protein synthesis after a few days have been observed in nondividing rose cell suspension cultures where the phenomenon could not be attributed to inhibitor breakdown or cell mutation (6). It has also been observed that actinomycin D infused in pear tissue resulted in incomplete inhibition of RNA synthesis (14).

In addition to the other advantages attributed to the use of cell cultures (relative homogeneity of cell population, uniform nutrition of cells, environmental control) we believe that a significant advantage is the ability to deprive the pear cells of hormones. In most systems used to study senescence, the level or the presence of endogenous hormones cannot be easily controlled. As observed by Cline (4) the effect of exogenous hormones can differ according to the endogenous concentration and may simply reflect changes in hormonal balance rather than primary action. The system for medium renewal described in this paper permits a continuous adjustment of media composition and the study of cellular senescence and death under controlled conditions.

Increases in protein and RNA synthesis have long been identified with the climacteric rise and final stages of senescence in many fruit, including the pear (14, 31). That is not to say that the processes of senescence and death in cultured pear fruit cells necessarily resemble or closely simulate what occurs in the senescent (ripening) pear. However, sufficient analogies may exist to render cell cultures especially useful in the study of fruit senescence.

Acknowledgment—We are grateful to Mr. Timothy Bos for capable and conscientious technical assistance.

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