

## THE EFFECT OF OXYGEN AND VITAMIN E ON THE LIFESPAN OF HUMAN DIPLOID CELLS IN VITRO

ARTHUR K. BALIN, DAVID B. P. GOODMAN, HOWARD RASMUSSEN, and VINCENT J. CRISTOFALO

From The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104 and the Department of Biochemistry, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19174

### ABSTRACT

Human diploid cells (WI-38) were serially subcultivated at partial pressures of oxygen ( $P_{O_2}$ ) ranging from 5.6 mm Hg to 608 mm Hg. At a  $P_{O_2}$  of 5.6 mm Hg, the number of doublings to phase out was less than that of control cells at a  $P_{O_2}$  of 137 mm Hg. Cultures grown at  $P_{O_2}$ 's of 24, 49, or 137 mm Hg grew at the same rate and phased out after a similar number of population doublings. Population lifespan was markedly shortened by chronic exposure to elevated  $P_{O_2}$ 's, a phenomenon that was, in part, reversible.

d- $\alpha$ -Tocopherol (10  $\mu$ g/ml or 100  $\mu$ g/ml) homogenized into the medium at each weekly subcultivation did not extend the lifespan of cells at reduced, ambient, or elevated oxygen tensions. These results indicate that neither oxygen toxicity nor free radical reactions play a significant role in limiting the lifespan of WI-38 cells grown in vitro under ambient oxygen tensions ( $P_{O_2}$  137 mm Hg).

The degradative changes associated with aging have been hypothesized to be due, in part, to free radical reactions (4, 25-27, 42, 51, 52) that are universal in living organisms (31). According to the free radical theory of aging, normal cellular function is ultimately limited by peroxidative damage which decreases the organisms' ability to respond adaptively to environmental change (26, 39, 52).

Evidence showing that peroxidative degradation of purified cellular components can be initiated in vitro by molecular oxygen, peroxidized lipids, or other free radical generating mechanism, lends support to this view (6, 7, 16, 50, 52).

However, the results of studies purporting to demonstrate animal lifespan extension after the addition of dietary antioxidants have been equivocal (8, 9).

Human diploid cells in culture present a unique

model system for examining the effects of oxygen and free radicals at the cellular level. These cultures have a finite lifespan in terms of the number of population doublings they can achieve (28, 29). The cultures can be serially subcultivated many times, but eventually growth slows, debris accumulates, and the population is lost. This degeneration has been interpreted as a manifestation of aging at the cellular level.

Several lines of evidence indicate that the aging changes observed in vitro may have relevance to those changes observed in vivo. For example, normal cells that are serially transplanted in vivo in syngeneic host animals also have a limited proliferative capacity (13, 14, 33, 49, 55). In cultures derived from human donors of different ages, there appears to be an inverse relationship between the lifespan of the culture and the age of the donor (23, 28, 35, 38, 43, 48).

As cultures of WI-38 cells age, the number of lysosomes and amorphous insoluble deposits increases (37, 44), and intracellular fluorescent deposits associated with the lysosomes and most pronounced in the nondividing cells accumulate (15). These deposits have been interpreted as being analogous to lipofuscin accumulated in the cells of aging animals and associated with lipid peroxidation (6, 15, 30, 51, 52).

Packer and Smith have reported that the addition of 10  $\mu\text{g/ml}$  or 100  $\mu\text{g/ml}$  of the antioxidant d-l- $\alpha$ -tocopherol extended the population doublings of WI-38 cells from 65 to 115 (40). In previous studies, we delineated the effect of oxygen on the growth and metabolism of WI-38 cells and demonstrated that these cells are exquisitely sensitive to growth inhibition by elevated partial pressures of oxygen (1-3). It was of interest, then, to investigate what changes in the lifespan of these cultures could be produced by alterations in ambient oxygen tension and to further evaluate the effects of vitamin E in the culture medium.

## MATERIALS AND METHODS

### *Cell Culture Procedure*

The human diploid cell line WI-38 (29) was obtained from Dr. L. Hayflick at Stanford University, and cells were grown in Eagle's minimal essential medium (MEM) (18) containing Earle's balanced salt solution (Auto Pow, Flow Laboratories, Rockville, Md.) and supplemented before autoclaving with vitamins as formulated for Eagle's basal medium (17). Immediately before use, the medium was supplemented with 2 mM L-glutamine (Flow Laboratories), 20 mM  $\text{NaHCO}_3$  (Microbiological Associates, Bethesda, Md.) and fetal bovine serum (FBS) (10% vol/vol; Flow Laboratories). No antibiotics were used.

Cultures were routinely grown at 37°C in 75  $\text{cm}^2$  sealed polystyrene flasks (Falcon Plastics no. 3024, Oxnard, Calif.) containing 40 ml of medium (46), and with a gas phase volume of 233  $\text{cm}^3$ . In one series of experiments, cells were grown in 65  $\text{cm}^2$  flint glass 8-ounce prescription bottles (Brockway Glass, Haddonfield, N. J.), tightly sealed with a red rubber sleeve stopper, and secured with rubber bands. These vessels were filled with 30 ml of medium and had a gas phase volume of 223  $\text{cm}^3$ . In both types of vessels, a 4.9-5.0 mm layer of medium covered the cell sheet.

Subcultivations were carried out weekly on confluent monolayers. The cells were released from the plastic by treatment with trypsin (0.25%) (Flow Laboratories) in  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free Eagle's MEM. After suspension in medium containing 10% FBS, the cells were counted and seeded into appropriate vessels at a density of  $1 \times 10^4$  cells/ $\text{cm}^2$ . All cell counts were done electronically,

using a Coulter Counter (Coulter Electronics, Hialeah, Fla.).

If the cultures were not confluent after 7 days of growth, they were refed every 7 days until confluence was reached or until phase-out. Phase-out was defined as the inability to achieve confluence after four feedings over a 4-wk period.

Population doublings were calculated in the standard way (11) by comparing cell counts per vessel at seeding with counts at confluence. The percentage of labeled nuclei was determined autoradiographically by the method of Cristofalo and Sharf (11). Cultures were monitored routinely for mycoplasma contamination by the method of Levine (36).

### *Routine Gassing Procedure*

The flasks were gassed to equilibrium both before and after the cells were seeded with analyzed certified standard gas mixtures obtained from Matheson Gas Products (East Rutherford, N. J.). These included 5%  $\text{CO}_2$ , balance  $\text{N}_2$ ; 5%  $\text{CO}_2$ , 5%  $\text{O}_2$ , balance  $\text{N}_2$ ; 5%  $\text{CO}_2$ , 20%  $\text{O}_2$ , balance  $\text{N}_2$ ; 5%  $\text{CO}_2$ , 50%  $\text{O}_2$ , balance  $\text{N}_2$ ; 5%  $\text{CO}_2$  and 95%  $\text{O}_2$ . A mixture of 10%  $\text{CO}_2$ , balance room air was obtained from Airco (Lodi, N. J.).

All vessels were incubated stationary at 37°C for 24 h to allow the cells to attach; the flasks were then placed (unless indicated) on a horizontally moving platform on a model 6250 Eberbach Shaker (Eberbach Corp., Ann Arbor, Mich.) adjusted to move through a 4-cm excursion cycle in 9 s. The gentle shaking insured that significant gas diffusion gradients would be minimized in the medium.

The oxygen tension of the medium of duplicate flasks was measured at weekly or biweekly intervals as described previously (2, 3), using a Blood Gas Analyzer (model 113, Instrumentation Laboratories, Lexington, Mass.). At an atmospheric pressure of 760 mm Hg, a 5.0%  $\text{CO}_2$ , 20.0%  $\text{O}_2$ , 75.0%  $\text{N}_2$ , gas mixture equilibrated in a flask with medium at 37°C would give a  $P_{\text{H}_2\text{O}}$  47 mm Hg,  $P_{\text{CO}_2}$  35.6 mm Hg,  $P_{\text{O}_2}$  142.6 mm Hg and  $P_{\text{N}_2}$  534.8 mm Hg.

The replicate lifespan experiments were performed with different sublines of WI-38 cells and by two investigators.

### *Recovery of Cells after Exposure to Elevated Oxygen Tensions*

The percentage of the cell population capable of initiating DNA synthesis after a 96-h exposure to an elevated (> atmos.) oxygen tension was determined autoradiographically. Young cells (88% labeled nuclei) were seeded at  $1 \times 10^4$  cells/ $\text{cm}^2$  in 2.0 ml of medium into two chamber Labtek vessels (Labtech, Inc., Westmont, Ill., 5.29  $\text{cm}^2/\text{well}$ ). The vessels were placed in stationary desiccators containing 3-5 cm of water to maintain humidity and equilibrated with 5%  $\text{CO}_2$ , 50%  $\text{O}_2$ , 45%  $\text{N}_2$  or 5%  $\text{CO}_2$ , 95%  $\text{O}_2$ . The oxygen tension in the desicca-

tors was monitored by withdrawing a sample of gas through a sealed septum covering the evacuation port. After 96 h at the elevated oxygen tension, the Labtek vessels were removed from the desiccators and placed in a 5% CO<sub>2</sub>, 95% room air ( $P_{O_2}$  137 mm Hg) incubator and [<sup>3</sup>H]thymidine was added to a final concentration of 0.1  $\mu$ Ci/ml or 2.5  $\mu$ Ci/ml (2 Ci/mmol). At the indicated times, the chambers were disassembled, and the slides rinsed three times in 37°C phosphate-buffered saline, and fixed for 30 min in 3:1 (vol/vol) of CH<sub>3</sub>OH:CH<sub>3</sub>CO<sub>2</sub>H followed by 5 min in CH<sub>3</sub>OH. At the completion of the experiment, all slides were simultaneously hydrolyzed and stained with Schiff's reagent, as described by Grove and Mitchell (24), and processed through autoradiography.

### Antioxidant Procedures

For all experiments involving vitamin E, d-l- $\alpha$ -tocopherol (Sigma Chemical Co., St. Louis, Mo.) was homogenized in complete medium (1 mg/ml), using an all stainless-steel Waring blender with Teflon washers (Eberbach Corp.). At each weekly subcultivation, freshly prepared vitamin E homogenate was added to the culture medium at a concentration of either 10  $\mu$ g per ml or 100  $\mu$ g per ml. Equivalent aliquots of homogenized medium without vitamin E were added to culture medium to control for homogenized media effects. Because of the slightly reduced attachment of cells initially exposed to vitamin E, all cultures were incubated without shaking.

Statistical significance was determined (unless otherwise indicated) by unpaired *t*-test analysis, using a separate variance formula with the *t* value for a given level of significance determined by averaging *t* values for  $n_1 - 1$  and  $n_2 - 1$  degrees of freedom (41). All experimental results are reported with the standard deviation of their mean ( $\pm$ SD).

## RESULTS

Fig. 1 illustrates the effect of oxygen tension on the lifespan of WI-38 cells. Cells serially subcultivated from population doubling level (PDL) 19 at  $P_{O_2}$ 's of 24 mm Hg, 49 mm Hg, or 137 mm Hg grew similarly and phased out after a similar number of population doublings, here 67-71. Cultures incubated at a  $P_{O_2}$  of 608 mm Hg did not proliferate. There were no differences in cell attachment between the various oxygen tensions throughout the cell lifespan (2).

Table I summarizes the results of a series of replicate experiments performed with different sublines of WI-38 cells. These results show that, in every case, although elevated oxygen tensions (> atmos.) poison WI-38 cells and markedly decrease their lifespan, there is no lifespan extension

when cells are serially subcultivated at lower partial pressure (< atmos.) of oxygen.

To determine the effect of very low oxygen tensions on lifespan, the cells were grown in sealed glass vessels that were kept stationary to maximize any oxygen diffusion gradients. Cultures that were serially subcultivated at a  $P_{O_2}$  of 5.6 mm Hg grew more slowly (2, 3) and phased out after fewer population doublings than control cells grown at a  $P_{O_2}$  of 119 mm Hg (Table II).

### Recovery after Exposure to Elevated Oxygen Tensions

The cultures were exposed to a  $P_{O_2}$  of  $341 \pm 34$  mm Hg for 7 days (week 1) and were then subcultivated and exposed to a  $P_{O_2}$  of  $137 \pm 9$  mm Hg or to a  $P_{O_2}$  of 341 mm Hg for various numbers of weeks (refeeding at weekly intervals) before being equilibrated with a  $P_{O_2}$  of  $137 \pm 9$  mm Hg serially for 3 additional weeks to allow recovery. Recovery was defined, in this experiment, as the ability to achieve enough cells in the flask to seed one daughter flask (at  $1 \times 10^4$  cells/cm<sup>2</sup>) and sufficient subsequent growth of the daughter flask to allow for subcultivation.

Cells incubated at a  $P_{O_2}$  of  $341 \pm 34$  mm Hg for 1 wk and then subsequently exposed to a  $P_{O_2}$  of  $137 \pm 9$  mm Hg recovered and were subcultivated for nearly as long as control cells never exposed to the higher oxygen tension (Table III).

Recovery of the cell population from prolonged exposure to elevated partial pressures of oxygen, although variable, was sharply limited if the cells were exposed to a  $P_{O_2}$  of 341 mm Hg for more than 2 wk. In addition, the lifespan of the cell populations that did recover after 5-7 weeks of exposure to  $P_{O_2} \geq 340$  was shorter than that of sister cultures never exposed to this elevated oxygen tension (Table III and Fig. 1).

Cells that had 95% labeled nuclei (LN) before a 7-day exposure to  $P_{O_2}$  341 mm Hg were found to have  $29 \pm 12\%$  ( $N = 17$ ) LN 24 h after this exposure. However, if these cells were allowed to recover at a  $P_{O_2}$  of 137 mm Hg for 7 days and were then subcultivated weekly, the percentage of LN increased, plateaued at about  $77 \pm 7\%$  LN ( $N = 18$ ) for weeks 2-4, and then gradually decreased over many subsequent weeks of growth as previously described by Cristofalo and Sharf (11).

To determine whether population recovery was due to the presence of a few oxygen-resistant clones in the population or whether recovery was

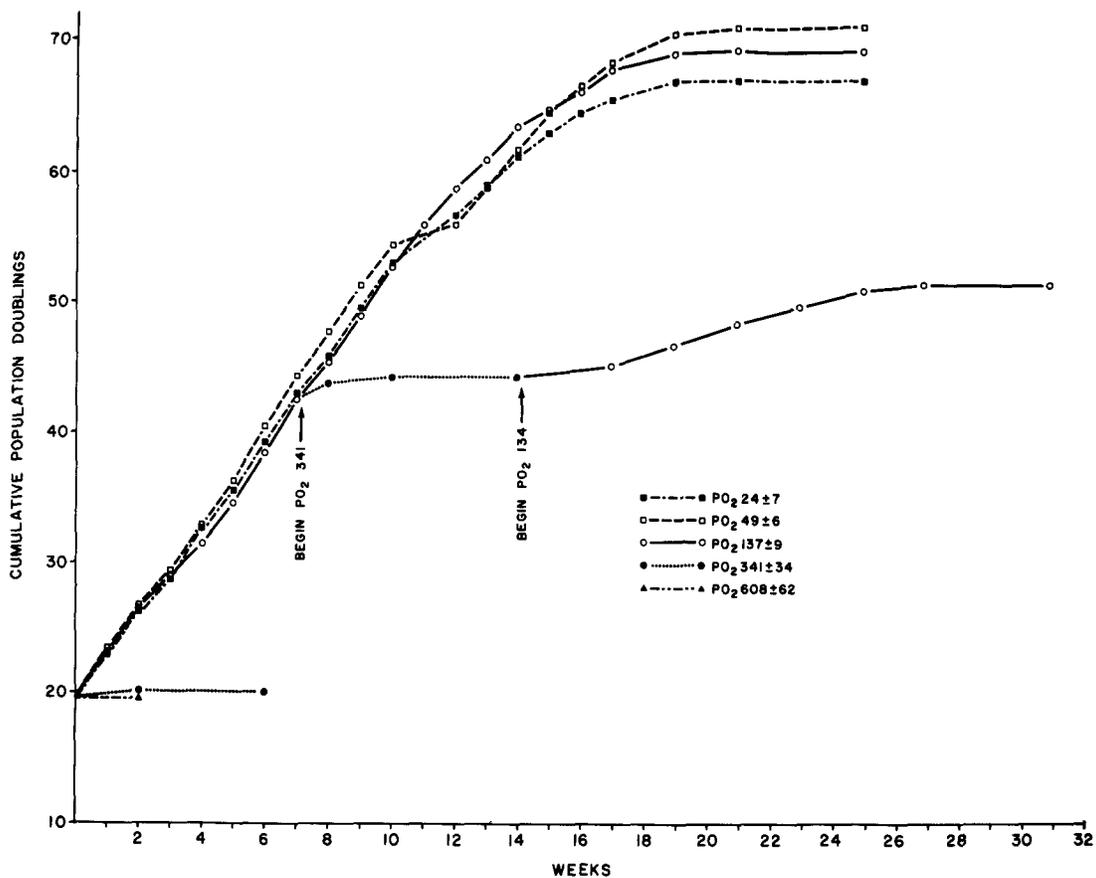


FIGURE 1 The effect of oxygen tension on the lifespan of human diploid cells. Cells were serially subcultivated as described in Materials and Methods at the indicated oxygen tension. This figure is an accumulative representation of the data whose endpoints are expressed in Table I, exp. 1 A and (for the recovery experiment) Table III.

TABLE I  
The Effect of Oxygen Tension on the Lifespan of WI-38 Cells

| Oxygen tension<br><i>mm Hg</i> ‡ | Population doubling level |            |      |       |           |       |           |       |           |  |
|----------------------------------|---------------------------|------------|------|-------|-----------|-------|-----------|-------|-----------|--|
|                                  | Exp 1*                    |            |      | Exp 2 |           | Exp 3 |           | Exp 4 |           |  |
|                                  | Begin                     | Phase-out§ |      | Begin | Phase-out | Begin | Phase-out | Begin | Phase-out |  |
| 24 ± 7                           | 19                        | 67         | 69   | 25    | 62        | 24    | 66        | 28    | 74        |  |
| 49 ± 6                           | 19                        | 71         | 72   | 25    | 59        | 24    | 74        | —     | —         |  |
| 137 ± 9                          | 19                        | 69         | 73   | 25    | 62        | 24    | 72        | 28    | 69        |  |
| 341 ± 34                         | 19.3                      | 20.0       | 20.1 | 25.0  | 25.4      | 34.5  | 35.5      | 28.5  | 29.4      |  |
| 608 ± 62                         | 19.3                      | 19.3       | 19.3 | 25.0  | 25.0      | 26.0  | 26.0      | 28.5  | 28.5      |  |
|                                  | 32.8                      | 32.8       | 32.8 |       |           |       |           |       |           |  |

The cells were serially subcultivated at the indicated oxygen tension and grown on a slowly shaking apparatus as described in Materials and Methods.

\* In Exp 1, A and B represent the work of two independent investigators carrying cells from the same initial culture to phase-out. Exp 1 A is illustrated in Fig. 1.

‡ Average  $P_{O_2}$  during growth period.

§ Phase-out was defined as the inability to achieve confluence after four feedings over a 4-wk period.

|| Standard deviation.

TABLE II  
The Effect of Low Oxygen Tension on the Lifespan of WI-38 Cells

| Oxygen tension<br><i>mm Hg</i> * | Population doubling level |            |       |           |       |           |
|----------------------------------|---------------------------|------------|-------|-----------|-------|-----------|
|                                  | Exp 1                     |            | Exp 2 |           | Exp 3 |           |
|                                  | Begin                     | Phase-out‡ | Begin | Phase-out | Begin | Phase-out |
| 119 ± 9§                         | 27                        | 61         | 26    | 54        | 24    | 65        |
| 5.6 ± 1.4 a                      | 27                        | 37         | 26    | 45        | 24    | 54        |
| 5.6 ± 1.4 b                      |                           |            | 26    | 35        | 24    | 55        |
| 5.6 ± 1.4 c                      |                           |            | 26    | 42        | 24    | 40        |
| 5.6 → 119¶                       |                           |            |       |           | 24    | 53        |

The cells were serially subcultivated at the indicated oxygen tension and grown without shaking in sealed glass prescription bottles as described in Materials and Methods.

\* Average  $P_{O_2}$  during growth period.

‡ Phase-out was defined as the inability to achieve confluence after four feedings over a 4-wk period.

§ Standard deviation.

|| Replicate experiments carried independently.

¶ Carried at  $P_{O_2}$  5.6 serially for 6 wk (38 PDL), then subcultivated at  $P_{O_2}$  119 until phase-out.

TABLE III  
Recovery of WI-38 Cell Populations after Prolonged Exposure to Elevated Oxygen Tensions

| Treatment at $P_{O_2}$ 341 ± 34 mm Hg | Recovery*                     | Lifespan of recovered cells PDL at phase-out experimental cultures (control‡) |
|---------------------------------------|-------------------------------|---|
| weeks                                 | no. recovered/no. experiments |   |
| 1                                     | 4/4                           | 54 (60); 52 (54); 51 (54);§   |
| 2                                     | 3/4                           | 45 (54); 52 (60);§  |
| 5-7                                   | 3/14                          | 52   (70); 52 (69);§  |

\* Cultures were exposed to a  $P_{O_2}$  of 341 mm Hg for the indicated time. All cultures were refed with fresh medium weekly. After week 1, the cultures were subcultivated because they were confluent. They did not become confluent again at  $P_{O_2}$  341 mm Hg. The ability of the culture to recover was tested by changing the atmosphere to  $P_{O_2}$  137 mm Hg and allowing the cultures to incubate at the lower oxygen tension for 3 additional weeks. Recovery was defined as the ability to achieve enough cells to seed one daughter flask ( $7.5 \times 10^6$  cells) and sufficient subsequent growth of the daughter flask to allow for subcultivation.

‡ Control: grown entirely at  $P_{O_2}$  137 ± 9 mm Hg.

§ In each case, one flask which met the criteria for recovery was lost to contamination during subsequent subcultivation procedure and before the lifespan was completed.

|| Treatment begun at PDL 43 (the weekly cumulative PDL of this subline is shown in Fig. 1, line A); in all other experiments, treatment begun at PDL 23-26.

general, we determined the fraction of the cell population that was able to initiate DNA synthesis after a 96-h exposure to elevated oxygen (Table IV).

The decline in microscope fields/1,000 cells ( $89 \pm 24$  vs.  $29 \pm 3$ ) reflects an increase in cell density and indicates that cell doubling occurred during recovery after exposure to  $P_{O_2}$  375 mm Hg. Less cell growth was obtained when 0.1  $\mu$ Ci/ml [ $^3$ H]thymidine was added to the culture for the

entire 72-h recovery period ( $64 \pm 11$ ) than when it was added only for the last 24 h of the period ( $29 \pm 3$ ), probably due to radiation damage (12).

To estimate what percentage of the population could initiate DNA synthesis, the cells were exposed to sufficient [ $^3$ H]thymidine (2.5  $\mu$ Ci/ml; 2 Ci/mmol) to prevent cell division. Under these conditions of high radioactivity for the entire 72-h

TABLE IV  
Cell Recovery at  $P_{O_2}$  137 mm Hg after 96-h Exposure to Elevated Oxygen Tension\*

| $P_{O_2}$ h 0-96 | Hours of continuous label‡ | [ $^3$ H]Thymidine $\mu$ Ci/ml | Cell density microscope fields/1,000 cells | Labeled nuclei§ |
|------------------|----------------------------|--------------------------------|--|-----------------|
|                  |                            |                                |  | %               |
| 375 mm Hg        | 96-120                     | 0.1                            | $89 \pm 24$                                | $37 \pm 8$      |
|                  | 120-144                    | 0.1                            | $47 \pm 17$                                | $68 \pm 6$      |
|                  | 144-168                    | 0.1                            | $29 \pm 3$                                 | $71 \pm 5$      |
|                  | 96-168                     | 0.1                            | $64 \pm 11$                                | $77 \pm 6$      |
|                  | 96-168                     | 2.5                            | $97 \pm 5$                                 | $63 \pm 6$      |
| 640 mm Hg        | 96-120                     | 0.1                            | $112 \pm 16$                               | $2 \pm 0.5$     |
|                  | 120-144                    | 0.1                            | $97 \pm 9$                                 | $23 \pm 5$      |
|                  | 144-168                    | 0.1                            | $85 \pm 19$                                | $53 \pm 3$      |
|                  | 96-168                     | 0.1                            | $136 \pm 31$                               | $34 \pm 4$      |
|                  | 96-168                     | 2.5                            | $127 \pm 21$                               | $34 \pm 3$      |

\* Three or four experiments in each category; 1,000-2,000 cells counted for each experiment.

‡ Cells were exposed to the elevated oxygen tension for 96 h before the atmosphere was changed to a  $P_{O_2}$  of 137 mm Hg to allow recovery. The medium was not changed. A continuous label of [ $^3$ H]thymidine was added at the indicated hour of the recovery period. The slides were fixed at the end of the labeling period. Thus, not all slides recovered for 72 h.

§ Cells in this experiment had completed 40% of their lifespan and had 88% labeled nuclei by the Cristofalo index.

|| Standard deviation.

period, the cells did not proliferate ( $97 \pm 5$  fields/1,000 cells vs.  $78 \pm 10$  fields/1,000 cells obtained from control cultures [data not shown in Table IV] after 97 h of exposure to  $P_{O_2}$  375 mm Hg and fixed before recovery was allowed). The data show that ~63% of the cells in this population were capable of initiating DNA synthesis (%LN) as compared to 88% for cells never exposed to elevated oxygen. Similarly, after 96-h exposure to 640 mm Hg, 34% (control 88%) of the cells were capable of initiating DNA synthesis in the first 72 h of recovery. Note that in this experiment the radiation damage induced by 0.1  $\mu$ Ci/ml of [ $^3$ H]thymidine, in conjunction with the effect of 96 h of a  $P_{O_2}$  of 640 mm Hg, was also sufficient to prevent the cells from dividing. We have previously shown that, after 168 h of exposure to a  $P_{O_2}$  of 600 mm Hg, 55–70% of the cells were capable of excluding erythrocin B dye (2). Therefore, it is possible that a larger fraction of the population would recover if a longer recovery time was allowed.

That substantial portions of the population were able to initiate DNA synthesis after exposure to elevated oxygen tensions suggests that we are not simply selecting a few resistant clones of cells. Population growth and metabolism during recovery from exposure to elevated oxygen tension has been reported in detail elsewhere (2).

#### Effect of Vitamin E and Oxygen Tension on Cell Lifespan

Vitamin E (d-l- $\alpha$ -tocopherol), an agent known to trap free radicals, has been reported to extend

the lifespan of WI-38 cells in culture (40). In our experiments, when vitamin E was first placed in the medium, about 14% ( $60 \pm 11\%$  vs.  $74 \pm 7\%$ ,  $N = 9$ , paired  $t$ -test  $P < 0.02$ ) fewer cells attached at 20 h in the vitamin E flasks than in controls that had an equivalent amount of homogenized medium added. This slightly decreased attachment was associated with a slight decrease in cell yield during the few weeks of cultivation under these conditions. However, cell attachment and cell yield equalized after 6–8 wk of subcultivation under the two conditions.

Table V summarizes representative experiments that show the effects of d-l- $\alpha$ -tocopherol on the lifespan of WI-38 cells at various oxygen tensions. Under our routine 5%  $CO_2$ :room air atmosphere, sister cultures carried serially in unsupplemented medium, or 10  $\mu$ g, or 100  $\mu$ g of d-l- $\alpha$ -tocopherol per ml showed no differences in lifespan. Similarly, cells carried serially under a  $P_{O_2}$  of 49 mm Hg or 137 mm Hg showed no differences in the PDL at phase-out with or without vitamin E. Cells grown under a  $P_{O_2}$  of  $341 \pm 34$  mm Hg were not protected by the addition of the antioxidant. Thus, in this series of experiments, vitamin E did not alter the lifespan of WI-38 cells in culture.

#### DISCUSSION

The fact that the lifespan of WI-38 cells in vitro is not extended by serial cultivation at  $P_{O_2}$ 's of 5.6 mm Hg, 26 mm Hg, or 50 mm Hg, suggests that neither oxygen toxicity nor free radical reactions play a significant role in delimiting the lifespan of

TABLE V  
The Effect of Vitamin E and Oxygen Tension on the Lifespan of WI-38 Cells

| Medium supplement                        | Population doubling level        |           |   |           |   |           |  |           |
|--|----------------------------------|-----------|---|-----------|---|-----------|--|-----------|
|  | Exp 1:<br>$P_{O_2}$ * 140 mm Hg† |           | Exp 2:<br>$P_{O_2}$ * 49 $\pm$ 6 mm Hg‡ |           | Exp 3:<br>$P_{O_2}$ * 137 $\pm$ 9 mm Hg |           | Exp 4:<br>$P_{O_2}$ * 341 $\pm$ 34 mm Hg |           |
|  | Begin                            | Phase-out | Begin                                   | Phase-out | Begin                                   | Phase-out | Begin                                    | Phase-out |
| —  | 18                               | 67        | 19                                      | 61        | 19                                      | 63        | 16.0                                     | 17.5      |
| 4 ml homogenized medium                  | 27                               | 69        | 37                                      | 64        | 35                                      | 65        | 16.0                                     | 17.5      |
| 10 $\mu$ g d-l- $\alpha$ -tocopherol/ml  | 24                               | 66        | 37                                      | 63        | 35                                      | 64        | 16.0                                     | 17.3      |
| 100 $\mu$ g d-l- $\alpha$ -tocopherol/ml | 18                               | 67        | 33                                      | 64        | 33                                      | 67        | 16.0                                     | 17.1      |

The cells were serially subcultivated at the indicated oxygen tension and grown without shaking as described in Materials and Methods. The medium supplement was freshly prepared and added at each weekly subcultivation. For each experiment, the supplemented cells were derived from the unsupplemented cultures in that experiment, at the indicated PDL (Begin).

\* Average  $P_{O_2}$  during growth period.

† Exp 1 was performed under our routine 5%  $CO_2$ :room air atmosphere and was not measured weekly.

‡ Standard deviation.

|| Phase-out was defined as the inability to achieve confluence after four feedings over a 4-wk period.

human diploid WI-38 cells in vitro under ambient oxygen tensions. This conclusion is further supported by the results showing that the addition of the free radical-trapping agent d-l- $\alpha$ -tocopherol at each subcultivation did not prolong lifespan. Furthermore, recovery after growth arrest resulting from exposure to elevated partial pressures of oxygen suggests that oxygen inhibits growth by a mechanism different from that responsible for the ultimate limitation on the proliferative capacity of the cell population.

If accumulated peroxidative damage played a significant role in limiting the lifespan of human diploid cells in culture, one would need to explain why similar damage does not limit the lifespan of tumor cell populations. It seems unlikely that the indefinite lifespan of HeLa or mouse LS cells could be due to a more efficient scavenging of free radicals since growth of these tumor cells is also very sensitive to inhibition by elevated partial pressures of oxygen (32, 45).

We previously showed (1, 2) that the population doubling time of exponentially growing WI-38 cells was minimal at a  $P_{O_2}$  from 26 to 50 mm Hg; and that although it increased by 15% at a  $P_{O_2}$  of 137 mm Hg, the final saturation densities were similar throughout this range of oxygen tension.

Ambient air has a  $P_{O_2}$  of 158; tracheal air in the human, a  $P_{O_2}$  of 149; alveolar air, a  $P_{O_2}$  of 100; arterial blood, a  $P_{O_2}$  of 95; and mixed venous blood, a  $P_{O_2}$  of 40 (34). The  $P_{O_2}$  venous blood overestimates the tissue  $P_{O_2}$  because gradients on the order of tens of mm Hg probably exist between the blood in the capillary systems and the sites of  $O_2$  reduction (19). Moreover, the rat continues breathing until the intracellular  $P_{O_2}$  in its brain falls below 0.2 mm Hg (5). From our studies, there appears to be a broad range of oxygen tensions in which the cells can live and divide normally, and the higher limit of this range is probably well above the  $P_{O_2}$  to which cells are normally exposed *in situ*.

Although we do not know how oxygen inhibits cell growth, we have established that such inhibition is due to some effect on the cells and not on the medium (2). In addition, the retardation of growth induced by  $P_{O_2}$ 's 290–560 mm Hg is not due to a generalized inhibition of cellular metabolism, because the glucose consumption and lactate production of these cells are markedly stimulated (2). Further, the cells have an extraordinary ability to recover from exposure to elevated oxygen tensions. Much of this population, not simply a

few resistant clones, is capable of initiating DNA synthesis in the first 72 h after a 96-h exposure to a  $P_{O_2}$  of 375 mm Hg (72%) or 640 mm Hg (40%). The lifespan of a population exposed to a  $P_{O_2}$  of 341 mm Hg for 1 wk appears to be the same as the lifespan of a culture never exposed to the elevated oxygen tension.

However, prolonged exposure (5–7 wk) to elevated oxygen tensions prevents most cultures from recovering. Interestingly, the lifespan of the cell populations that did recover was shorter than that of control cells. It is not possible to interpret the significance of the length of the recovered populations' lifespan until we know how large a fraction of the cell population recovers after this prolonged exposure.

Packer and Smith (40) reported that the addition of 10  $\mu$ g/ml or 100  $\mu$ g/ml d-l- $\alpha$ -tocopherol to WI-38 cells at each subcultivation extended the in vitro lifespan of the cells from 65 to 115 PDL. Furthermore, they reported that a brief exposure to d-l- $\alpha$ -tocopherol (from the 45th to 73rd PDL) conferred the same effect as continual exposure. Using methodology similar to theirs, we have been unable to reproduce this result. Packer and Smith used a single substrain of WI-38 cells; we used multiple substrains of WI-38 cells and repeated the experiment over 15 times. Finally, Packer and Smith (40) did indicate in a subscript to their paper that their experiments were begun with one lot of serum and that, after their cells had gone 90–105 PDL, a new serum lot was started and the cells phased out. They suggest that there was some unique property of that one serum lot which explains their results. We noted a 14% poorer attachment when cells were initially exposed to vitamin E; however, attachment equalized after 6–8 wk of growth (20 population doublings) on the antioxidant. However, even if (a) this attachment difference persisted for the entire cell lifespan, (b) PDL was calculated using a ratio of cell attachment to cell yield rather than the conventional method of cells inoculated to cell yield, and (c) the weekly cell yield was identical in the vitamin E and control flasks (rather than the slightly better initial growth in the control flasks), the maximum additional population doublings attributed to the antioxidant-treated cultures over those attributed to control cultures would be less than six. Thus, even allowing for these remote possibilities, there was no lifespan extension in the cells that received the antioxidant.

Other investigators have found either no effect

or a mild inhibitory effect of d-l- $\alpha$ -tocopherol on fibroblast growth in vitro (Chinese hamster fibroblasts, V79 lung fibroblasts) on medium containing glucose (10). In rats, antioxidant compounds provided a partial protection from the toxicity of a  $P_{O_2}$  of 4,560 mm Hg but did not protect against the toxicity of the more moderate  $P_{O_2}$  of 760 mm Hg (20).

We previously discussed the conflicting reports in the literature concerning the lowest oxygen tension that could sustain cell growth (2). We demonstrated that at a  $P_{O_2}$  of  $7.8 \pm 3.5$  mm Hg, WI-38 cell growth was markedly inhibited. This inhibition was accompanied by a markedly decreased  $O_2$  consumption and both increased glucose consumption and lactate production. Serial cultivation of these cells at a  $P_{O_2}$  of  $5.6 \pm 1.4$  mm Hg (this report) demonstrated some variability in the PDL at phase-out, but in no case did the lifespan equal or exceed that of control cells. These observations are of interest since Warburg (53) hypothesized that hypoxia or any respiratory injury sufficient to decrease oxygen consumption and increase glycolytic metabolism was the basis for the etiology of neoplasia. Although controversial (54), this point of view has been proposed by others such as Goldblatt and Cameron (21) who reported transformation of rat fibroblasts upon intermittent exposure to low oxygen tensions. In a subsequent publication, however, dealing with the malignant transformation of fibroblasts and epithelial cells exposed to hypoxic conditions, it was reported that both the cultures intermittently exposed to  $N_2$  and the control cultures transformed (22). Goldblatt et al. (22) explained this phenomenon by postulating that the control cells, which had free access to oxygen, transformed because of inadequate diffusion of atmospheric oxygen into the liquid medium of the stationary flask. In this regard, it is important to point out that, after more than 10 years of WI-38 cell cultivation, there have not been any published reports of a spontaneous transformation. In addition, Sanford was unable to induce malignant transformation of cells in vitro by intermittent anaerobiosis (47). Furthermore, it should be emphasized that WI-38 cells, serially carried at a  $P_{O_2}$  of 5.6 mm Hg where oxygen consumption is minimal and glycolytic metabolism is markedly increased, do not acquire an indefinite lifespan in vitro.

The authors wish to acknowledge the expert technical assistance provided by Ms. Dori Green.

This work was supported by U. S. Public Health Service grants AG-00378 from the National Institute of Aging, CA-14345 from the National Cancer Institute, and NR-202-005 from the Office of Naval Research. A. K. Balin is a trainee of the Medical Scientist Training Program, National Institutes of Health (GM-02046).

This study was presented, in part, by Dr. Arthur Balin in partial fulfillment of the requirements for the Ph.D. A preliminary account of some of this work has been presented at the 1975 Tissue Culture Association Meeting.

Received for publication 6 October 1976, and in revised form 22 February 1977.

## REFERENCES

- BALIN, A. K., D. G. GOODMAN, H. RASMUSSEN, and V. J. CRISTOFALO. 1974. The effect of oxygen tension on the growth and aging of WI-38 cells. *In Vitro (Rockville)*. **10**:384.
- BALIN, A. K., D. G. GOODMAN, H. RASMUSSEN, and V. J. CRISTOFALO. 1976. The effect of oxygen tension on the growth and metabolism of WI-38 cells. *J. Cell. Physiol.* **89**:235-250.
- BALIN, A. K., D. G. GOODMAN, H. RASMUSSEN, and V. J. CRISTOFALO. 1976. Atmospheric stability in cell culture vessels. *In Vitro (Rockville)*. **12**:687-692.
- BOENIG, H. 1966. Free radicals and health: indicators for a unifying concept. *J. Am. Geriatr. Soc.* **14**:1211-1220.
- CHANCE, B., P. COHEN, F. JOBIS, and B. SCHÖNER. 1962. Intracellular oxidation-reduction states in vivo. *Science (Wash. D. C.)*. **137**:499-508.
- CHIO, K. S., U. REISS, B. FLETCHER, and A. L. TAPPEL. 1969. Peroxidation of subcellular organelles: formation of lipofuscinlike fluorescent pigments. *Science (Wash. D. C.)*. **166**:1535-1536.
- CHIO, K. S., and A. L. TAPPEL. 1969. Inactivation of ribonuclease and other enzymes by peroxidizing lipids and by malonaldehyde. *Biochemistry*. **8**:2827-2833.
- COMFORT, A. 1974. The position of aging studies. *Mech. Ageing Dev.* **3**:1-31.
- COMFORT, A., I. YOHUTSKY-GORE, and K. PATHMANATHAN. 1971. Effect of ethoxyquin on the longevity of C3H mice. *Nature (Lond.)*. **229**:254-255.
- CORWIN, L. M., and L. P. HUMPHREY. 1972. Vitamin E: substrate dependent growth effect on cells in culture. *Proc. Soc. Exp. Biol. Med.* **141**:609-612.
- CRISTOFALO, V. J., and B. B. SHARF. 1973. Cellular senescence and DNA synthesis. *Exp. Cell Res.* **76**:419-427.
- CRISTOFALO, V. J. 1976. Thymidine labelling index as a criterion of aging in vitro. *Gerontology*. **22**:9-27.
- DANIEL, C. W., K. B. DEOME, J. T. YOUNG, P. B. BLAIR, and L. J. FAULKIN, JR. 1968. The in vivo

- lifespan of normal and preneoplastic mouse mammary glands: a serial transplantation study. *Proc. Natl. Acad. Sci. U. S. A.* **61**:53-60.
14. DANIEL, C. W., and L. J. T. YOUNG. 1971. Influence of cell division on an aging process. Lifespan of mouse mammary epithelium during serial propagation in vivo. *Exp. Cell Res.* **65**:27-32.
  15. DEAMER, D. W., and J. GONZALES. 1974. Autofluorescent structures in cultured WI-38 cells. *Arch. Biochem. Biophys.* **165**:421-426.
  16. DESAI, I. D., P. L. SAWANT, and A. L. TAPPEL. 1964. Peroxidative and radiation damage to isolated lysosomes. *Biochim. Biophys. Acta.* **86**:277-285.
  17. EAGLE, H. 1955. The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure. *J. Exp. Med.* **102**:595-600.
  18. EAGLE, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Wash. D. C.)*. **130**:432-437.
  19. FORSTER, R. E. 1965. Oxygenation of the tissue cell. *Ann. N. Y. Acad. Sci.* **117**:730-735.
  20. GERSCHMAN, R., D. L. GILBERT, and D. CACCA-MISE. 1958. Effect of various substances on survival times of mice exposed to different high oxygen tensions. *Am. J. Physiol.* **192**:563-571.
  21. GOLDBLATT, H., and G. CAMERON. 1953. Induced malignancy in cells from rat myocardium subjected to intermittent anaerobiosis during long propagation *in vitro*. *J. Exp. Med.* **97**:525-552.
  22. GOLDBLATT, H., L. FRIEDMAN, and R. L. CHECHNER. 1973. On the malignant transformation of cells during prolonged culture under hypoxic conditions *in vitro*. *Biochem. Med.* **7**:241-252.
  23. GOLDSTEIN, S., J. W. LITTLEFIELD, and J. S. SOELDNER. 1969. Diabetes mellitus and aging: diminished plating efficiency of cultured human fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **64**:155-160.
  24. GROVE, G. L., and R. B. MITCHELL. 1974. DNA microdensitometry as a measure of cycling-noncycling activity in aged human diploid cells in culture. *Mech. Ageing Dev.* **3**:235-240.
  25. HARMAN, D. 1956. Aging: A theory based on free radical and radiation chemistry. *J. Gerontol.* **11**:298-300.
  26. HARMAN, D. 1962. Role of free radicals in mutation, cancer, aging, and the maintenance of life. *Radiat. Res.* **16**:753-763.
  27. HARMAN, D. 1969. Prolongation of life: role of free radical reactions in aging. *J. Am. Geriatr. Soc.* **17**:721-735.
  28. HAYFLICK, L., and P. S. MOORHEAD. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**:585-621.
  29. HAYFLICK, L. 1965. The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.* **37**:614-636.
  30. HENDLEY, D. D., A. S. MILDVAN, M. C. RE-PORTER, and B. L. STREHLER. 1963. The properties of isolated human cardiac age pigment. II. Chemical and enzymatic properties. *J. Gerontol.* **18**:250-259.
  31. ISENBERG, I. 1964. Free radicals in tissue. *Physiol. Res.* **44**:487-517.
  32. KILBURN, D. G., M. D. LILLY, D. A. SELF, and F. C. WEBB. 1969. The effect of dissolved oxygen partial pressure on the growth and carbohydrate metabolism of mouse LS cells. *J. Cell Sci.* **4**:25-37.
  33. KROHN, P. L. 1962. Heterochronic transplantation in the study of ageing. *Proc. Roy. Soc. Ser. B.* **157**:128-147.
  34. LAMBERTSON, C. 1974. The atmosphere and gas exchanges with the lungs and blood. In *Medical Physiology*. V. Mountcastle, editor. C. V. Mosby Company, St. Louis, Mo. 13th edition. **2**:1372-1374.
  35. LEGUILLY, Y., M. SIMON, P. LENOIR, and M. BOUREL. 1973. Long-term culture of human adult liver cells: morphological changes related to *in vitro* senescence and effect of donor's age on growth potential. *Gerontologia.* **19**:303-313.
  36. LEVINE, E. M. 1972. Mycoplasma contamination of animal cell cultures: a simple rapid detection method. *Exp. Cell Res.* **74**:99-109.
  37. LIPETZ, J., and V. J. CRISTOFALO. 1972. Ultrastructural changes accompanying the aging of human diploid cells in culture. *J. Ultrastruct. Res.* **39**:43-56.
  38. MARTIN, G. M., C. A. SPRAGUE, and C. J. EPSTEIN. 1970. Replicative lifespan of cultivated human cells. *Lab. Invest.* **23**:86-92.
  39. PACKER, L., D. W. DEAMER, and R. L. HEATH. 1967. Regulation and deterioration of structure in membranes. *Adv. Gerontol. Res.* **2**:77-120.
  40. PACKER, L., and J. R. SMITH. 1974. Extension of the lifespan of cultured normal human cells by Vitamin E. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4763-4767.
  41. POPHAM, W. J. 1967. *Educational Statistics: Use and Interpretation*. Harper and Row, New York. 129-163.
  42. PRYOR, W. A. 1971. Free radical pathology. *Chem. and Eng. News* **49**:34-51.
  43. RHEINWALD, J. G., and H. GREEN. 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell.* **6**:331-344.
  44. ROBBINS, E., E. M. LEVINE, and H. EAGLE. 1970. Morphological changes accompanying senescence of cultured human diploid cells. *J. Exp. Med.* **131**:1211-1222.
  45. RUECKERT, R. R., and G. C. MUELLER. 1960. Effect of oxygen tension on HeLa cell growth. *Cancer Res.* **20**:944-949.
  46. RYAN, J. M., B. B. SHARF, and V. J. CRISTOFALO. 1975. The influence of culture medium volume on cell density and lifespan of human diploid fibro-

- blasts. *Exp. Cell Res.* **91**:389-392.
47. SANFORD, K. K. 1965. An attempt to induce malignant transformation of cells *in vitro* by intermittent anaerobiosis. *J. Natl. Cancer Inst.* **35**:719-725.
  48. SCHNEIDER, E. L., and Y. MITSUI. 1976. Examination of the relationship between *in vitro* cellular aging and *in vivo* human age. *Proc. Natl. Acad. Sci. U. S. A.* **73**:3584-3588.
  49. SIMINOVITCH, L., J. E. TILL, and E. A. McCULLOCH. 1974. Decline in colony-forming ability of marrow cells subjected to serial transplantation into irradiated mice. *J. Cell. Comp. Physiol.* **64**:23-31.
  50. TAPPEL, A. L. 1965. Free radical lipid peroxidation damage and its inhibition by vitamin E and selenium. *Fed. Proc.* **24**:73-78.
  51. TAPPEL, A. L. 1968. Will antioxidant nutrients slow aging processes? *Geriatrics.* **23**:97-105.
  52. TAPPEL, A. L. 1973. Lipid peroxidation damage to cell components. *Fed. Proc.* **32**:1870-1874.
  53. WARBURG, O. 1956. On the origin of cancer cells. *Science (Wash. D. C.)*. **123**:309-314.
  54. WEINHOUSE, S., O. WARBURG, D. BURK, and A. C. SCHADE. 1956. On respiratory impairment in cancer cells. *Science (Wash. D. C.)*. **124**:267-272.
  55. WILLIAMSON, A. R., and B. A. ASKONAS. 1972. Senescence of an antibody-forming cell clone. *Nature (Lond.)*. **238**:337-339.