

## OXYGEN-SENSITIVE STAGES OF THE CELL CYCLE OF HUMAN DIPLOID CELLS

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### ABSTRACT

We had established that growth of human diploid WI-38 cells is reversibly inhibited by elevated partial pressures of oxygen ( $P_{O_2}$ ) and we were interested in determining where in the cell cycle growth was delayed. A technique combining cytospectrophotometry and autoradiography was used to determine cell cycle parameters. Confluent cells that were subcultivated and exposed to a  $P_{O_2}$  of  $365 \pm 8$  mm Hg were delayed primarily after DNA synthesis but before metaphase. At a  $P_{O_2}$  of  $590 \pm 35$  mm Hg, most cells did not initiate DNA synthesis, and the few that did, failed to complete the process. When exponentially growing cells that had already begun DNA synthesis were exposed to a  $P_{O_2}$  of  $590 \pm 35$  mm Hg, they accumulated after completing DNA synthesis but before initiating mitosis. The rate at which [ $^3$ H]thymidine was incorporated into DNA was inversely correlated with oxygen tension ( $P_{O_2}$  of 135–590 mm Hg). These results suggest that the process most sensitive to oxygen causes cells to be delayed after DNA synthesis but before metaphase. Slightly higher  $P_{O_2}$ 's were needed to inhibit the initiation of DNA synthesis. Further, the rate of DNA synthesis is decreased by elevated oxygen tensions.

**KEY WORDS** oxygen · cell cycle · aging · human diploid cells · cell proliferation

The precise nature of oxygen's interaction in cellular metabolism is not understood, although it is clear that oxygen participates in several fundamental cellular processes. In addition to oxygen's performing a central role in respiration, oxygen-induced cellular lesions have been suspected of being involved in cancer, aging, differentiation, and evolution (5, 6, 12, 17, 20, 23, 25).

In our previous studies, we showed that elevated oxygen tensions reversibly inhibited the

growth of human diploid cells (2–4). Oxygen-induced inhibition of cellular proliferation was not due to a generalized oxidation of sulfhydryl enzymes, because under growth-inhibiting conditions glycolysis was markedly stimulated (4). By systematically investigating oxygen's effect on cell growth and metabolism, we are attempting to elucidate the various aspects of oxygen's function in cellular economy. In this report, we describe the biological basis of oxygen-induced inhibition of cell growth by determining whether oxygen-inhibited cells accumulate at random points or at particular stages of the cell cycle.

## MATERIALS AND METHODS

### Cell Culture Procedure

The human diploid cell line WI-38 (18) was obtained from Dr. L. Hayflick (Stanford University). Details of the cell cultivation procedures that are summarized here have been described previously (2, 7).

Cells were grown in autoclavable Eagle's minimal essential medium (MEM) (11) containing Earle's balanced salt solution (Auto Pow, Flow Laboratories, Rockville, Md.). Before autoclaving, this medium was supplemented with an additional mixture of vitamins as formulated for Eagle's Basal Medium (10). Immediately before use, the medium was supplemented with L-glutamine (2 mM) (Flow Laboratories),  $\text{NaHCO}_3$  (20 mM) (Microbiological Associates, Bethesda, Md.), and fetal bovine serum (FBS) (10% vol/vol; Flow Laboratories). No antibiotics were used.

Cultures were routinely grown without shaking at 37°C in 75-cm<sup>2</sup> sealed polystyrene flasks (BioQuest, BBL & Falcon Products, Becton Dickinson & Co., Cockeysville, Md., no. 3024) containing 40 ml of media, in 5%  $\text{CO}_2$ , 95% air. Routine 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 electronically counted with a Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.) and inoculated into appropriate vessels at a density of  $1 \times 10^4$  cells/cm<sup>2</sup>. Cultures were monitored routinely for mycoplasma contamination by the method of Levine (22). The percentage of labeled nuclei was determined autoradiographically by the method of Cristofalo and Sharf (7). Young WI-38 cells (population doubling level 22-30; >90% labeled nuclei) were used in this study.

### Experimental Technique

Cells were inoculated at  $1 \times 10^4$  cells/cm<sup>2</sup> in 2.0 ml of medium into each chamber of two-chamber Labtek vessels (Labtech, Inc., Westmont, Ill., 5.29 cm<sup>2</sup>/well). The height of the medium was 5 mm above the cell surface. We maintained humidity in the growth vessels by placing them either in a humidified 5%  $\text{CO}_2$ :95% air incubator (37°C), or in desiccators that contained 3-5 cm of water and were equilibrated with 5%  $\text{CO}_2$ , 50%  $\text{O}_2$ , 45%  $\text{N}_2$ , or 5%  $\text{CO}_2$ , 95%  $\text{O}_2$  (Matheson Gas Products, East Rutherford, N. J.). The desiccators were placed in an incubator at 37°C.

The oxygen tension in the desiccators and incubator was monitored before each manipulation by withdrawing a sample of gas through a sealed septum covering the evacuation port, as described by Balin et al. (4), using an Instrumentation Laboratory Model 113 Blood Gas Analyzer (Instrumentation Laboratory, Inc., 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 equi-

brated in a flask with medium at 37°C would give a  $P_{\text{H}_2\text{O}}$  of 47 mm Hg,  $P_{\text{CO}_2}$  of 35.6 mm Hg,  $P_{\text{O}_2}$  of 142.6 mm Hg, and  $P_{\text{N}_2}$  of 534.8 mm Hg.

The cells were grown for various time intervals as indicated in the text. 1 h before harvest, [<sup>3</sup>H]thymidine was added to the vessels to a final concentration of 2.5  $\mu\text{Ci/ml}$  (2 Ci/mmol, New England Nuclear, Boston, Mass.). The cells were maintained at the specified oxygen tension during the thymidine pulse.

### Oxygen's Effect on Cell Growth

Growth characteristics of the WI-38 cell system under exposure to various  $P_{\text{O}_2}$ 's have been described elsewhere (2). Representative curves showing growth over the range of oxygen tensions employed in the present study are illustrated in Fig. 1, and aid interpretation of the data presented.

### Slide Processing and Feulgen Staining

After the indicated number of hours of growth, the chambers were disassembled, and the slides were rinsed three times in 37°C phosphate-buffered saline, fixed for 30 min in 3:1  $\text{CH}_3\text{OH}:\text{CH}_3\text{CO}_2\text{H}$ , then for 5 min in  $\text{CH}_3\text{OH}$ , and allowed to air dry. At the completion of the experiment, all slides were simultaneously hydrolyzed for 45 min in 5 N HCl at 25°C (8), stained with Schiff's reagent for 2 h, then washed three times in freshly prepared bleach (0.4 g of  $\text{Na}_2\text{S}_2\text{O}_5$ , 1.0 ml of concd. HCl, 100 ml of  $\text{H}_2\text{O}$ ). They were then rinsed in water and dehydrated through a graded ethanol series.

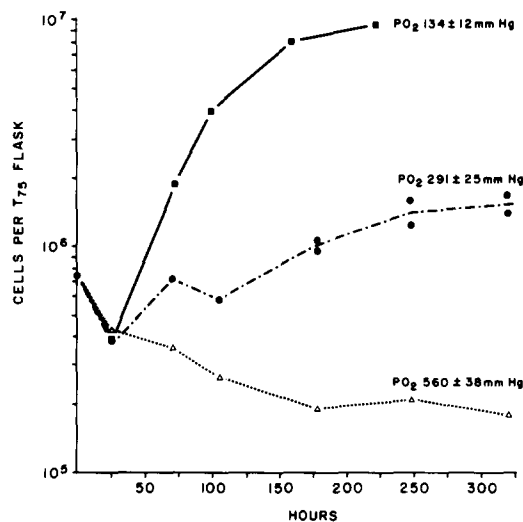


FIGURE 1 The growth of WI-38 cells during exposure to various partial pressures of oxygen. The numbers associated with each curve represent the measured  $P_{\text{O}_2}$  of the media at harvest. Cells were seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> and were grown in 75-cm<sup>2</sup> polystyrene vessels. Methodological details for this figure are described by Balin et al. (2).

After being air-dried, they were processed for autoradiography as described by Cristofalo and Sharf (7). After development, the slides were dehydrated through a graded ethanol-xylene series (16), and cover slips were mounted with Harleco synthetic resin (Harleco, Philadelphia, Pa.). For photomicrography, slides were fixed for 5 min in  $\text{CH}_3\text{OH}$  and stained with May-Grünwald-Giemsa reagent (5 min), then dehydrated and mounted as previously described. The slides were photographed through a Leitz Orthoplan microscope on a Kodak photomicrography color film no. 2483 with a Kodak 8A filter over a tungsten light source.

The percentage of labeled (five grains per nucleus over background) cells was determined by counting 500 nuclei at random. The relative grain intensity of the labeled nuclei was determined on a randomly selected population ( $n > 75$ ) using the Vickers M85 scanning microdensitometer (Vickers Instruments, York, England) at a wavelength ( $\lambda = 460$  nm) where absorption due to the Feulgen-stained DNA was minimal. The DNA content of the unlabeled interphase nuclei was determined in randomly selected cells ( $n > 100$ ,  $\lambda = 565$  nm) using the scanning microdensitometer as described by Goldstein (13, 14).

The percentage of mitotic (mid-late prophase through telophase) cells was determined by counting  $>1,000$  nuclei at random. The DNA content of mitotic cells provided an internal DNA standard of 4C DNA. In some experiments, peripheral erythrocytes of a 15-month-old white Leghorn hen (2–4 pg DNA/cell) (1) were processed with the experimental slides to provide an external DNA standard that could be used to confirm the diploid DNA content in cases where no mitotic cells could be found.

The DNA content of a confluent cell population (93% diploid, see Results) grown under 5%  $\text{CO}_2$ , 20%  $\text{O}_2$ , 75%  $\text{N}_2$  was measured 7 days after seeding. Cells were trypsinized, pelleted by centrifugation (500 g, 5 min, 4°C) and washed three times with  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free phosphate-buffered saline (4°C). The cells were resuspended, an aliquot was counted electronically, and the DNA content was determined by the Hinegardner (19) modification of the method of Kissane and Robbins (21) using calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) as a standard. All experimental results are reported with the standard deviation of the mean ( $\pm$  SD).

## RESULTS

### *Oxygen-Sensitive Phases in the Cell Cycle of WI-38 Cells Exposed to Elevated $P_{\text{O}_2}$ 's at Seeding*

Cells grown for 72 h under normal atmospheric oxygen tension ( $P_{\text{O}_2}$  of  $138 \pm 5$  mm Hg) proliferated actively (2); when cells were exposed to

$[^3\text{H}]\text{dT}$  from hour 71–72, 45% of the population was observed to be synthesizing DNA and 2.5% of the population was in mitosis (Fig. 2a). The DNA content of the unlabeled interphase nuclei fell into two populations: 40% had a DNA content of 2C and 13% had a DNA content of 4C (Fig. 2a). At a higher  $P_{\text{O}_2}$  ( $373 \pm 7$  mm Hg), cells grew very slowly over the 72-h period (4) (Fig. 1); and from hour 71–72, only 13% of the population was engaged in DNA synthesis, while only 0.1% of the population was engaged in mitosis (Fig. 2b, Table I). However, a marked percentage of the population contained a 4C content of DNA (47% at 72 h) while 35% of the cells were found to have a 2C DNA content. Cells exposed to a  $P_{\text{O}_2}$  of  $649 \pm 33$  mm Hg do not proliferate (2) (Fig. 1). Only 1% of these cells synthesized DNA during the pulse from hour 71–72, and  $<0.1\%$  were in mitosis (Table I). After 72 h, 70% of the population contained a 2C complement of DNA, only 10% contained a 4C complement of DNA, and 19% of the cells had an intermediate content of DNA, referred to as "3C." Occasionally, at all oxygen tensions, a few cells ( $<2\%$ ) were encountered with an 8C content of DNA (Fig. 2b and c).

To illuminate these findings, we compared the time-course of the change in the distribution of cells through the cell cycle for the various oxygen tensions. Immediately after a confluent population (grown at a  $P_{\text{O}_2}$  of 138 mm Hg) was subcultivated (time 0), the population distribution was approx. 93% 2C, 5% 4C, 2% labeled (after a 1-h  $[^3\text{H}]\text{thymidine}$  pulse),  $<0.1\%$  mitosis (Fig. 3); and the DNA content was  $7.69 \pm 1.9$   $\mu\text{g}$  DNA/ $10^6$  cells ( $n = 19$ ). The cells retained this distribution for 14–18 h before the percentage of the population undergoing DNA synthesis began to increase. The population was parasynchronous for the first 36 h at a  $P_{\text{O}_2}$  of 138 mm Hg, but became random by 48 h (data not shown). A larger percentage of the population initiated DNA synthesis by 24 h when cells were grown at a  $P_{\text{O}_2}$  of 138 mm Hg (57%) (Fig. 2a and 3a) than when cells were grown at a  $P_{\text{O}_2}$  of 365 mm Hg (45%) (Fig. 2b and 3b). This decrease at a  $P_{\text{O}_2}$  of 365 mm Hg indicates that the transition from the pre-DNA synthetic phase ( $G_0$ ,  $G_1$ ) to DNA synthesis (S) is slightly delayed or inhibited at the higher oxygen tension. In addition, the percentage of cells synthesizing DNA decreased during the 72-h incubation period under a  $P_{\text{O}_2}$  of 365 mm Hg from 45% at hour 24 to 13% by hour 72 (Fig.

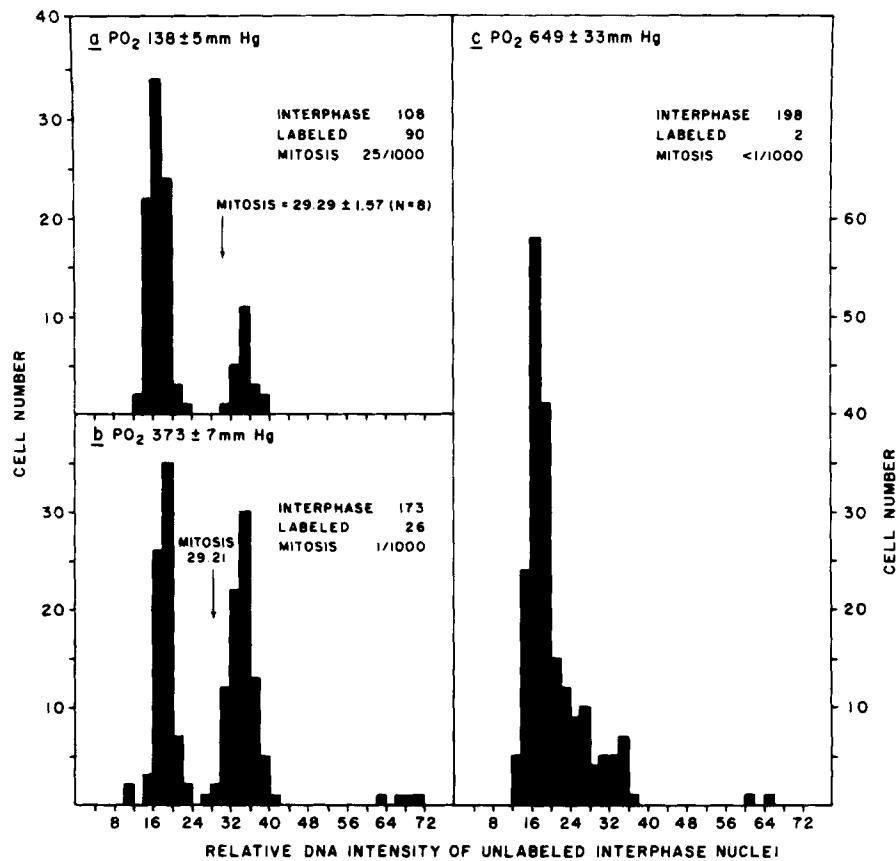


FIGURE 2 The distribution through the cell cycle of a cell population grown at various oxygen tensions for 72 h. The scanning microdensitometer was used to determine the relative DNA content of the unlabeled interphase nuclei. Young confluent WI-38 cells were subcultivated and incubated at the indicated oxygen tensions at hour 0, pulsed with [ $^3$ H]thymidine from hour 71-72, fixed at hour 72, then Feulgen-stained and processed through autoradiography as described (see Materials and Methods). 200 cells were selected randomly and scored as labeled (five grains over background), mitotic, or interphase cells. The relative DNA content of the unlabeled interphase nuclei is plotted in the histogram. The haploid content of DNA is referred to as "1C." Metaphase mitotic figures are used to indicate the 4C DNA peak. Absorption due to the condensed chromosomes of the metaphase mitotic cells usually falls at the lower end of the 4C distribution. Chick erythrocytes (2.4 pg/DNA nucleus) had a relative DNA intensity of  $4.74 \pm 0.56$  ( $n = 6$ ).

3b). Thus, it seems that the delay in initiation of DNA synthesis became more pronounced with increasing time of exposure to the elevated oxygen tension. The most striking finding, however, was the increasing proportion of the population with a 4C complement of DNA (47% by 72 h) (Fig. 3b). This indicates that at a  $P_{O_2}$  of 365 mm Hg most of the population was delayed after completing DNA synthesis but before initiating mitosis.

The cell population exposed to a  $P_{O_2}$  of  $590 \pm 35$  mm Hg immediately after subcultivation was markedly delayed in the initiation of DNA synthe-

sis: 73% of the cells contained a 2C content of DNA and 20% incorporated [ $^3$ H]thymidine from hour 23-24, whereas when cells were exposed to a  $P_{O_2}$  of 138 mm Hg for the same length of time, 35% contained a 2C content of DNA and 58% incorporated [ $^3$ H]thymidine (Fig. 3a and c). During continued exposure to a  $P_{O_2}$  of 590 mm Hg, there was no further initiation of DNA synthesis; at hours 48 and 72, 70% of the population retained a 2C DNA content (Fig. 3c), and no increase in cell number was noted (2) (Fig. 1). Furthermore, the proportion of the population

TABLE I  
The Time-Course of the Change in Mitotic Index during Growth at Various Oxygen Tensions

$P_{O_2}$	Mitosis (% of total population)*			
	Hours after subcultivation			
	1	24	48	72
$138 \pm 5$ †	<0.1 (3)§	$1.3 \pm 0.2$ (2)	$1.9 \pm 0.2$ (2)	$2.2 \pm 0.4$ (4)
$365 \pm 8$	<0.1 (3)	$0.2 \pm 0.2$ (2)	$0.4 \pm 0.3$ (3)	$0.1 \pm 0.1$ (4)
$590 \pm 35$	<0.1 (3)	$0.2 \pm 0.2$ (2)	0.1 (2)	<0.1 (4)

\* Mitotic index from experiment depicted in Fig. 3 (see legend, Fig. 3). Index determined from counting >1,000 random cells per slide.

† Standard deviation

§ nos. in parentheses = no. of replicate slides counted for this experiment.

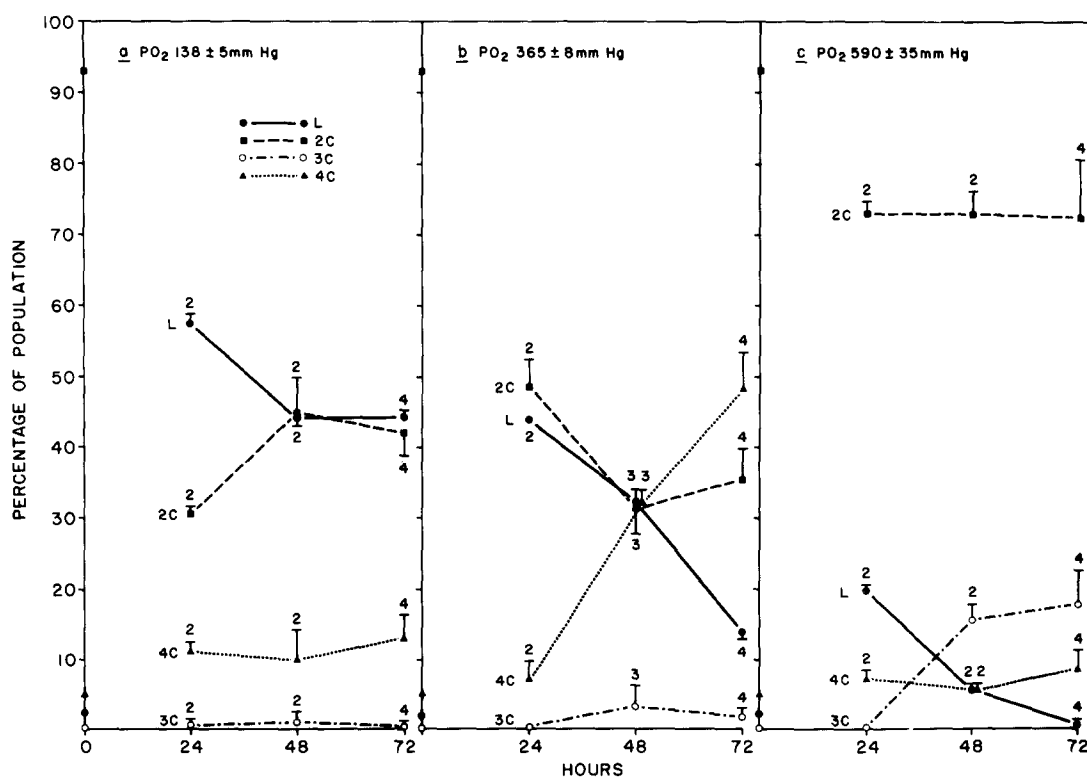


FIGURE 3 The time-course of the change in the interphase population distribution during growth of young WI-38 cells exposed to various oxygen tensions at seeding. Cells were seeded from confluent cultures and exposed to the indicated oxygen tension immediately upon subcultivation. The cells were pulsed with [<sup>3</sup>H]thymidine for 1 h immediately before fixation (see Materials and Methods). The population distribution at time 0 represents the distribution of young confluent WI-38 cells for the first 14–18 h after subcultivation. The number associated with each point is the number of replicate slides counted; error bars indicate standard deviation. The data in this figure were derived from histograms (e.g., Fig. 2). The percentage of labeled nuclei (L) was determined from 500 randomly selected cells, and the interphase cell distribution was determined from >100 random measurements. Cells with other DNA contents (such as 8C) represented <2% of the population and were not plotted on these graphs. The change in mitotic index of the population in this experiment is presented in Table I.

that was capable of initiating DNA synthesis (20%) aborted after an incomplete attempt, as shown by the progressive decline in the proportion of the population incorporating [<sup>3</sup>H]thymidine and the corresponding increase in unlabeled 3C cells (Fig. 3c). Unlabeled cells did not move through the cell cycle for there was no increase in 4C interphase cells (Fig. 3c) or mitotic cells (Table I). Thus, when confluent cells were subcultivated and exposed to a  $P_{O_2}$  of 590 mm Hg,

initiation of effective DNA synthesis was inhibited.

#### *Nuclear Morphology of Cells Exposed to Oxygen*

The morphology of unstained, growing WI-38 cells exposed to various  $P_{O_2}$ 's has been detailed elsewhere (2). Fig. 4 depicts the nuclear morphology of cells exposed from seeding to  $P_{O_2}$ 's of 138 mm Hg (Fig. 4a), 373 mm Hg (Fig. 4b), and 649

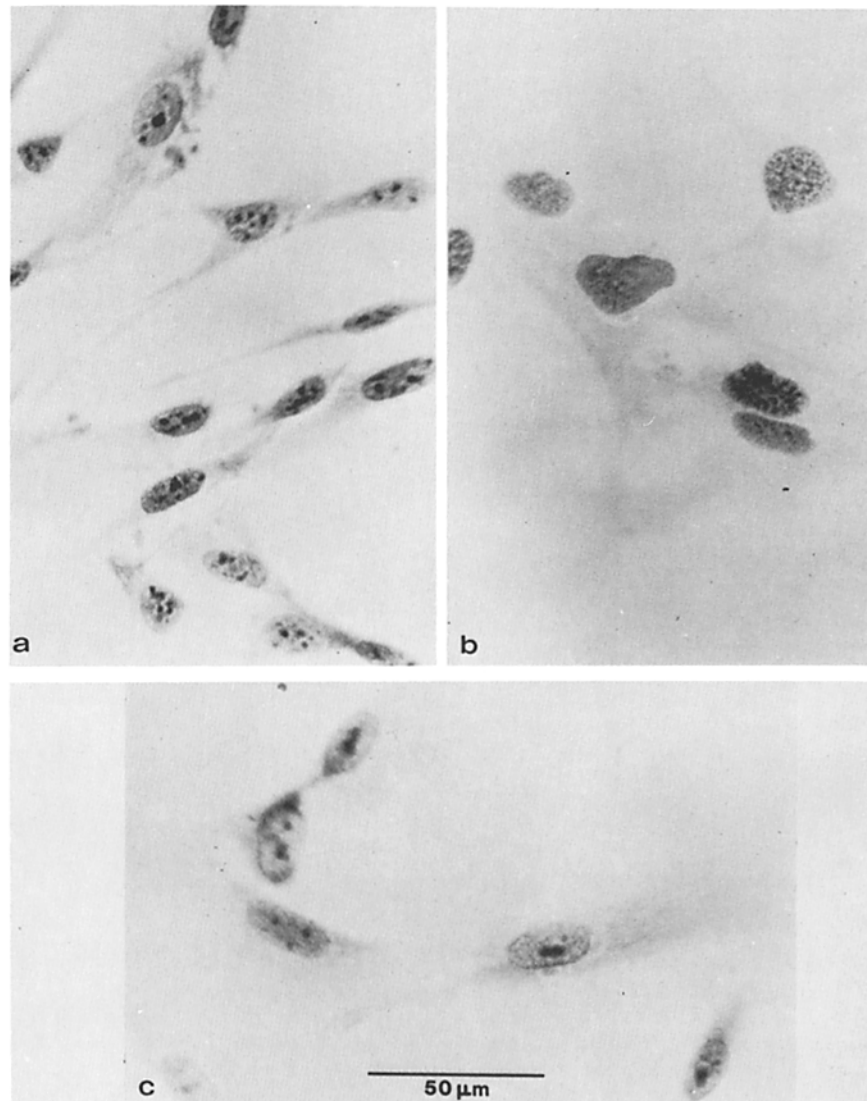


FIGURE 4 Representative fields of young WI-38 cells subcultivated and grown for 72 h under (a)  $P_{O_2}$  of  $138 \pm 5$  mm Hg; (b)  $P_{O_2}$  of  $373 \pm 7$  mm Hg; and (c)  $P_{O_2}$  of  $649 \pm 33$  mm Hg. May-Grünwald-Giemsa staining.

mm Hg (Fig. 4c) and grown for 72 h. A large proportion of the population exposed to a  $P_{O_2}$  of 373 mm Hg has large nuclei; these cells have a 4C DNA content and are representative of the 4C arrested cells (Fig. 4b). In particular, morphologically the nuclei appear to be in interphase or early prophase. Thus, the cell cycle delay in cells exposed to a  $P_{O_2}$  of 373 mm Hg appears after DNA synthesis but before late prophase. Cells exposed to a  $P_{O_2}$  of 649 mm Hg from seeding are primarily inhibited from initiating effective DNA synthesis and do not accumulate the large 4C DNA nuclei (Fig. 4c).

#### Oxygen-Sensitive Phases in the Cell Cycle of WI-38 Cells Exposed to Elevated Oxygen Tension after the Onset of DNA Synthesis

To examine the effect of oxygen on actively proliferating cells, a confluent population was subcultivated and grown at a  $P_{O_2}$  of 138 mm Hg for 24 h before exposure to the experimental oxygen tensions. In this experiment, 50% of the

exponentially growing cells kept at a  $P_{O_2}$  of 138 mm Hg incorporated [ $^3$ H]thymidine: 40% had a 2C content of DNA, and 10% had a 4C DNA content (Fig. 5a). As in the previous experiment, there was a gradual accumulation of cells with a 4C DNA content when the atmosphere was changed to a  $P_{O_2}$  of 353 mm Hg (Fig. 5a vs. 5b). When cells were allowed to begin DNA synthesis before exposure to a  $P_{O_2}$  of 592 mm Hg, the proportion of them engaged in DNA synthesis after the increase in  $P_{O_2}$  to 592 mm Hg increased from 0 to 16 h, whereas at lower  $O_2$  tensions the proportion of the population undergoing DNA synthesis declined gradually from hour 0 (Fig. 5a-c). These data suggest that cells exposed to a  $P_{O_2}$  of 592 mm Hg needed more time to complete DNA synthesis than did cells at lower  $P_{O_2}$ 's. The absence of 3C cells at a  $P_{O_2}$  of 592 mm Hg indicates that cells initiating DNA synthesis eventually completed the process and accumulated as nonmitotic 4C cells (Fig. 5c). The 4C accumulation (evidence of a delay in initiation of mitosis) was more pronounced at a  $P_{O_2}$  of 592 mm Hg

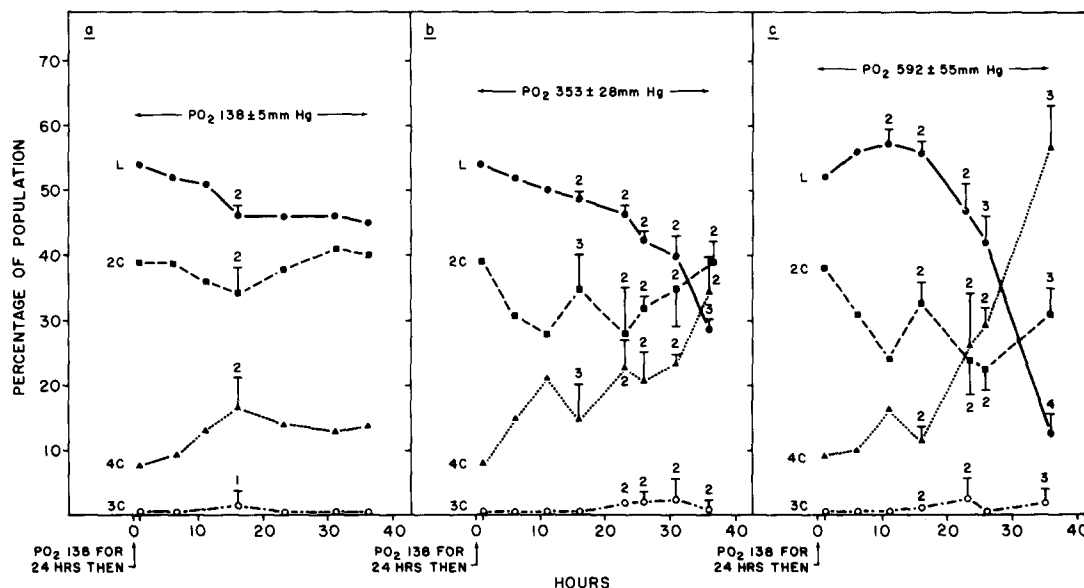


FIGURE 5 The time-course of the change in the interphase population distribution during growth of young WI-38 cells exposed to various oxygen tensions after the onset of exponential growth. Cells were seeded from confluent cultures and allowed to enter the cell cycle by incubation at a  $P_{O_2}$  of 138 mm Hg for 24 h. The atmosphere was changed as indicated and designated time "0." The percentage of labeled nuclei was determined from 500 randomly selected cells, and the interphase cell distribution was determined from >100 random measurements. The data in this figure were derived from histograms (e.g., Fig. 2). Cells with other DNA contents (such as 8C) represented <2% of the population and were not plotted on these graphs. The number associated with each point is the number of replicate slides counted; error bars indicate standard deviation. The mitotic index of the cell populations in this experiment is not illustrated, but it represented <4% of the population.

than at a  $P_{O_2}$  of 353 mm Hg (Fig. 5b vs. 5c). These findings show that when cells began DNA synthesis before being exposed to a  $P_{O_2}$  of 592 mm Hg, they were capable of completing DNA synthesis but were unable to initiate mitosis. In addition, after a 24-h exposure to a  $P_{O_2}$  of 592 mm Hg, the proportion of the population incorporating [ $^3$ H]thymidine declined without a corresponding decline in 2C cells, indicating that the delay in the initiation of DNA synthesis was more pronounced with increasing time of exposure to the elevated oxygen tension.

#### Oxygen and the Rate of DNA Synthesis

Further evidence that elevated oxygen tension may decrease the rate of DNA synthesis is found in an analysis of the relative grain intensity of labeled cells. The relative grain intensity of labeled cells reflects the amount of [ $^3$ H]thymidine incorporated into DNA during the 1-h pulse pe-

riod. Analysis of histograms of the relative grain intensity of labeled cells reveals an inverse correlation between the rate of [ $^3$ H]thymidine incorporation and the oxygen tension (Fig. 6). The inverse correlation between the rate of [ $^3$ H]thymidine incorporation and oxygen tensions in these cells can be seen in Fig. 6. Furthermore, the decrease in the rate of [ $^3$ H]thymidine incorporation began shortly (by hour 5) after exposure to the elevated oxygen tension and was greatest when the oxygen tension was highest ( $P_{O_2}$  592  $\pm$  55) (Fig. 7).

The average grain intensity of the labeled nuclei of cells incubated at a  $P_{O_2}$  of 353 or 592 mm Hg tended either to remain constant or to decrease over a 72-h incubation. Yet the average grain intensity tended to increase between hours 48 and 72 at a  $P_{O_2}$  of 138 mm Hg (data not shown). We cannot provide a certain explanation for this increase; however, it is possible that, after 48 h, cell growth at a  $P_{O_2}$  of 138 mm Hg was sufficient

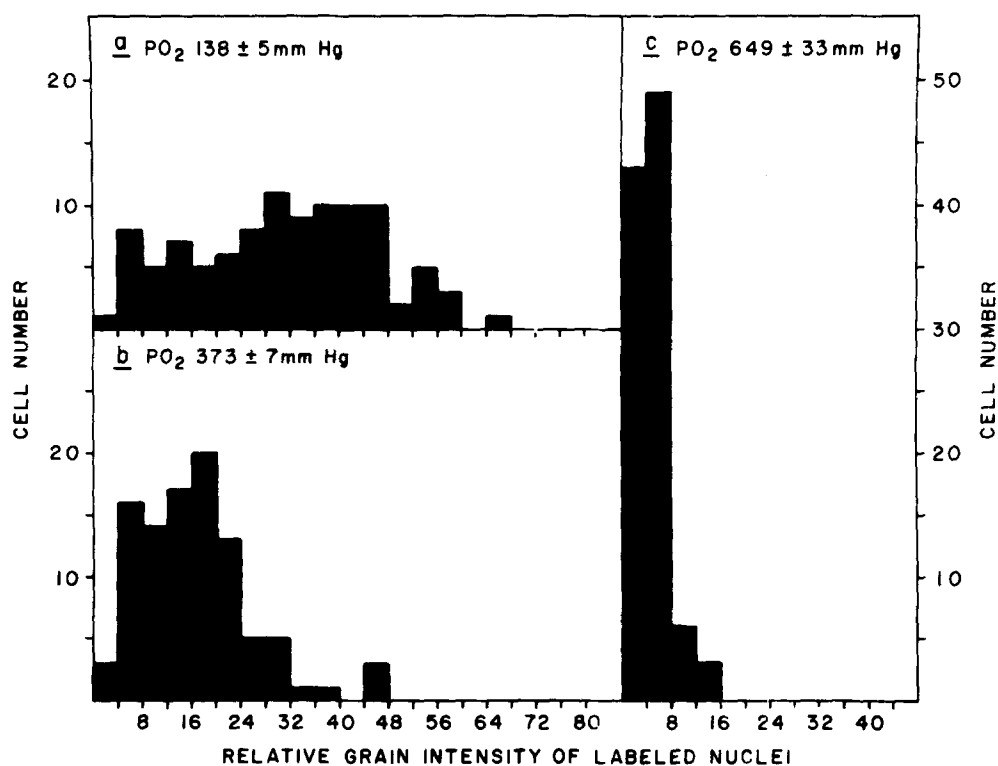


FIGURE 6 The relative grain intensity of a population of 101 randomly selected labeled nuclei after a 48-h exposure to various oxygen tensions. Young confluent WI-38 cells were subcultivated and incubated at the indicated oxygen tensions at hour 0, pulsed with [ $^3$ H]thymidine from hour 47-48, fixed at hour 48, then Feulgen-stained and processed through autoradiography as described (see Materials and Methods). The relative grain intensity of the labeled nuclei (at least five grains over background) was measured by the scanning microdensitometer. Unlabeled nuclei had a relative intensity of  $1.9 \pm 2.0$  ( $n > 100$ ).



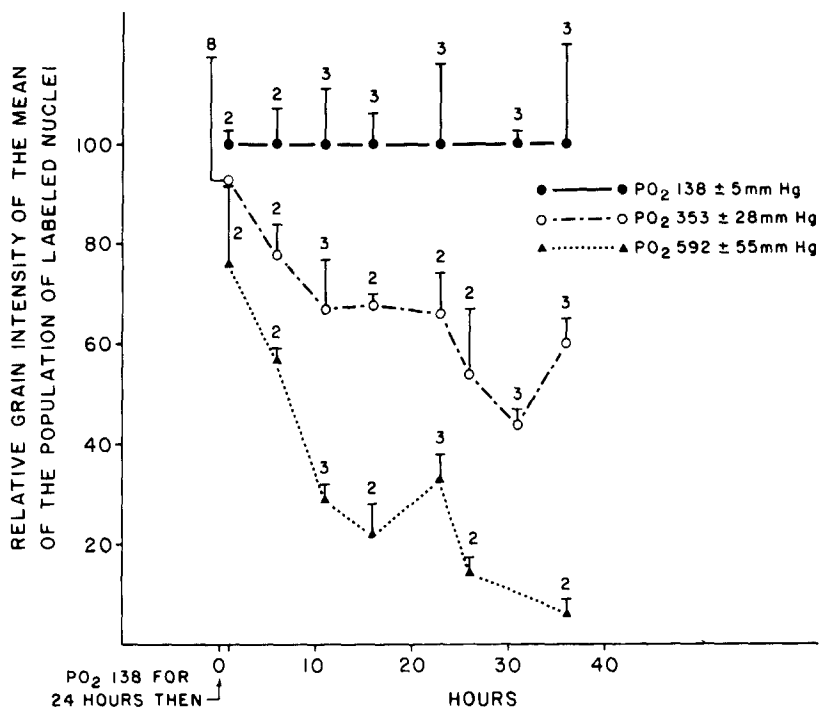


FIGURE 7 The time-course in the change of the relative grain intensity of the mean of the population of labeled nuclei (five grains over background) after exposure to various oxygen tensions. Cells were seeded from confluence and allowed to enter the cell cycle by incubation at a  $P_{O_2}$  of 138 mm Hg for 24 h. The atmosphere was changed at time 0 as indicated, and the slides were pulsed with [ $^3$ H]thymidine for 1 h immediately before fixation. The mean values of a population of labeled nuclei ( $n > 75$ ) were determined. The mean values of replicate experiments were averaged and normalized so that the value for the control population equaled 100. The values were corrected for the contribution due to the absorption of the unlabeled nuclei. The number associated with each point indicates the number of replicate slides counted. Error bars indicate  $\pm 1$  SD.

to reduce the endogenous medium thymidine concentration.

## DISCUSSION

In our previous studies, we demonstrated that elevated  $P_{O_2}$ 's reversibly inhibit the growth of the human diploid cell strain, WI-38. In this report, we sought to determine whether cells inhibited by elevated oxygen tension occur at random points or accumulate at particular stages of the cell cycle. Three stages of the cell cycle were found in which oxygen-inhibited cells accumulate. The most striking data we obtained showed that cells exposed to a  $P_{O_2}$  of 350 mm Hg at seeding were delayed primarily after DNA synthesis but before mitosis (Figs. 2b, 3b, 4, and Table I). These findings indicate that the site most sensitive to oxygen causes a delay in  $G_2$  (or early prophase). Another site, less sensitive to oxygen, delayed resting ( $G_0$ )

cells from initiating DNA synthesis. Confluent cells that were subcultivated and exposed to a  $P_{O_2}$  of 365 mm Hg were partially delayed in entry to DNA synthesis since under this elevated  $P_{O_2}$  the percentage of cells engaged in DNA synthesis at hour 24 (45%) was lower than the percentage of cells synthesizing DNA in the control group (57%) (Fig. 3a and b). Furthermore, confluent cells that were freshly subcultivated and exposed to a  $P_{O_2}$  of 590 mm Hg were primarily inhibited from entering DNA synthesis. It is unlikely that these results are related to selective detachment of cells from the substratum. Cell attachment at various  $P_{O_2}$  levels has been reported in our previous work (2) and has been shown to be independent of  $P_{O_2}$ .

A third site sensitive to oxygen inhibition reduced the rate of [ $^3$ H]thymidine incorporation into the DNA of cells that were exposed to

elevated oxygen tensions during DNA synthesis (Fig. 5c, 6, and 7). The reduction was due either to a decrease in thymidine transport and/or utilization, or to a decreased rate of DNA synthesis. On the basis of our results, it is not possible to completely resolve these alternative explanations. However, oxygen did not block thymidine transport and utilization completely (independent of the effect that slows the rate of DNA synthesis to zero), since no cells synthesized DNA and proceeded through the cell cycle without incorporating [<sup>3</sup>H]thymidine. We know this because there were virtually no 3C cells in a population incubated under a  $P_{O_2}$  of 365 mm Hg (Fig. 3b) and no increase in 4C cells under incubation at a  $P_{O_2}$  of 592 mm Hg (Fig. 3c). On the other hand, the rate of DNA synthesis was slowed to zero for most cells exposed to a  $P_{O_2}$  of 592 mm Hg for 72 h (Fig. 3c). It should be noted that the stage of the cell cycle at which oxygen-inhibited cells accumulate is not necessarily the stage in which the oxygen-sensitive sites are located.

Drew et al. (9) reported that an atmosphere of 95%  $O_2$  inhibited the DNA synthesis of exponentially growing HeLa cells. He and his associates monitored DNA synthesis by determining the relative grain intensity of autoradiographs prepared by pulse labeling exponentially growing HeLa cells with [<sup>3</sup>H]thymidine or [<sup>131</sup>I]deoxyuridine. They also estimated, on the basis of the percent labeled nuclei, that  $G_2$  was prolonged by 1 h.

In another study, Gerschman et al. (12) suggested that oxygen poisoning and X-ray damage occur through a common free radical mechanism. Indeed, elevated  $P_{O_2}$ 's have been shown to cause chromosomal aberrations and breaks (6) as has radiation damage (27). Several researchers have investigated the effects of radiation on the cell cycle of mammalian cells (24, 26, 28). Cells appear to be most sensitive to radiation when they are in  $G_2$ -M with a secondary, but usually less sensitive, point at the  $G_0$ -S interphase. Most cells appear to be relatively resistant to lethal irradiation during S, although radiation seems to slow the rate of DNA synthesis (24). Thus there is a similarity between the stages of the cell cycle sensitive to radiation and those in which oxygen-inhibited cells accumulate.

In previous studies, we have shown that growth inhibition by elevated oxygen tension is, at least in part, reversible upon re-exposure to lower oxygen tensions (2). It may be possible to use oxygen to

obtain a reversibly inhibited  $G_2$ -enriched population.

Our previous investigation of oxygen's effect on cellular lifespan tested the applicability of the free radical theory of aging to a diploid cell culture derived from human lung fibroblasts. We found that although elevated oxygen tensions poison WI-38 cells and markedly decrease their lifespan, their lifespan is not extended by serial subcultivation at low  $P_{O_2}$ 's ( $P_{O_2}$ 's of 6, 26, and 50 mm Hg), even with the addition of the free radical trapping agent vitamin E (4). These findings suggest that oxygen toxicity and free radical reactions are not significant in delimiting the lifespan of human diploid cells under atmospheric oxygen tensions (4). The present study provides an additional indication that aging and oxygen toxicity occur by different mechanisms in the human diploid cell system.

Both confluence and aging in WI-38 cell populations are associated with an accumulation of cells with a 2C content of DNA (15, 16, 29). The present report demonstrates that the site most sensitive to inhibition by elevated oxygen tension causes cells to be delayed with a 4C content of DNA. Therefore, it does not appear that oxygen toxicity is the process responsible for growth delay during cellular aging.

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