Effect of Oxygen Tension on HeLa Cell Growth*

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SUMMARY

High oxygen concentrations were shown to have a powerful growth-inhibitory effect on HeLa cultures. Initially there was a generalized reduction in the rate of cell division and in the biosynthesis of DNA, RNA, and protein, a shift in glucose metabolism to a completely anaerobic pattern, and an accompanying acceleration in the rate of glucose utilization. After about one generation time cell division ceased completely. Finally, after about 36 hours the lethal effect of the oxygen lesion was expressed as a failure of polymer biosynthesis and rapid loss of reproductive powers.

The optimal oxygen concentration for HeLa cells growing in Eagle's medium is 20–30 per cent, or about that concentration found in air.

While numerous observations have been made on the general toxicity of high oxygen tensions in animal cell systems, relatively little attention has been directed to the biochemical analysis of such oxygen effects in quantitative tissue culture systems. In studies with lung and intestinal cells from chick embryos, Jones and Bonting (8) observed that an atmosphere containing 100 per cent oxygen resulted in a high rate of glycolysis and ultimately was lethal to the cells. Similarly, Cooper, Burt, and Wilson (3) reported briefly that monkey kidney cells did not proliferate well in the presence of oxygen concentrations greater than 20 per cent.

It is the purpose of this communication to describe the changes in the growth rate, metabolism, basic cell composition, and viability of HeLa cells which resulted from exposure to various oxygen tensions. At oxygen tensions above 30 per cent, cell division was blocked first, and subsequently the syntheses of DNA, RNA, and protein were also halted as a state of unbalanced growth resulted.

MATERIALS AND METHODS

The experiments described in this paper were performed with a human carcinoma cell[1] (HeLa) grown in our laboratory during the last 4 years on Eagle's HeLa medium (4) containing 10 per cent whole bovine serum[2] and 2×10^{-5} M inositol. Earle's balanced salt solution (5) was employed as the electrolyte base, and cultures were routinely gassed with 5 per cent CO_2 in air unless otherwise stated. This medium is hereafter referred to as BEHM. Stock cultures were prepared by seeding 50,000 cells/ml of BEHM in Roux bottles, and the cells were permitted to grow for 3 days to assure an exponentially growing population.

Experiments were carried out either in 16 × 150-mm. test tubes or in 3-oz. prescription bottles as indicated. Replicate test tube cultures were prepared by inoculating the indicated number of cells in 1.0 ml. of BEHM. After being gassed with 5 per cent CO_2 in air, the cultures were incubated at an angle of 5° from the horizontal in Seelye racks[3] at 37° C. for 24 hours to permit attachment of the cells to the glass. Following the replacement of the medium with 4.0 ml. of fresh medium, an experiment was initiated by gassing the cultures with the indicated mixtures of oxygen and nitrogen. The tubes were flushed for 10 seconds with a flow rate of 3500 cc/min. Gas mixtures were prepared with the aid of calibrated flowmeters[4] by mixing 5 per cent CO_2 in oxygen with 5 per cent

* This work was supported by a grant from the Alexander and Margaret Stewart Trust Fund, Grant No C-1897 from the United States Public Health Service, and a grant from the T. Evans Brittingham Estate.

[1] The HeLa cell strain used in these experiments was obtained originally from Dr Gilbert Chang, State Laboratory of Hygiene, Madison, Wis.

Received for publication January 18, 1960.

[2] The serum was prepared in our laboratory from freshly defibrinated blood obtained from the Oscar Mayer Packing Plant, Madison, Wis. Sterilization was effected by passage through 03 Selas porcelain filters.

[3] Seelye Craftsman, 984 Central Ave, Minneapolis, Minn.

[4] Matheson Chemical Company, Joliet, Illinois. Flowmeters #204 and 205 were used.

CO_2 in nitrogen at the indicated ratio; all gas mixtures thereby contained 5 per cent CO_2.

The viability of cells was determined by plating known numbers of trypsinized cells into BEHM according to the methods described by Puck *et al.* (12). After removal of the medium, the monolayer to be assayed for plating efficiency was washed briefly with 0.9 per cent saline and then incubated at 37° C. in 10 ml. of 0.05 per cent trypsin (obtained from Nutritional Biochemicals Corporation under the designation 1-300) in saline A (14). After incubation for 15 minutes at 37° C. an equal volume of BEHM was added, and the cells were dispersed by repeated aspiration and discharge from a pipette. With due caution this method of preparation yields monodispersed suspensions of HeLa cells with a viability of 80–100 per cent when assayed for ability to form macroscopic colonies from single cells. Aliquots of such cell suspensions were plated into a final volume of 4.5 ml. BEHM in 60-mm. petri dishes and incubated under a water-saturated atmosphere of 5 per cent CO_2 in air for 7–10 days, at which time the colonies were fixed with Bouin's fixative and stained with Giemsa stain for counting.

For the determination of the cell compositions the culture medium was decanted, and the cell sheets were washed 4 times with cold saline to remove residual medium.[5] The cell sheets were then washed successively for 5-minute intervals with 4 per cent perchloric acid, 50 per cent ethanol, 95 per cent ethanol, and absolute ethanol at room temperature. Finally they were rinsed with absolute ethanol and dried with ether. The dry sheet of cell residue was then dissolved in 2.0 ml. of 88 per cent formic acid, and suitable aliquots were desiccated and analyzed directly. Ribonucleic acid (RNA) was determined by the method of Ceriotti (2), deoxyribonucleic acid (DNA) by a modified fluorometric procedure of Kissane and Robins (9), and protein by Oyama and Eagle's (13) modification of the Lowry method. When only protein was determined, the cell sheet was dissolved directly after the saline washes in the alkaline copper reagent and analyzed.

A modified anthrone procedure was used to determine glucose in the culture medium (10). Lactic acid was determined by the method of Barker and Summerson (1). Catalase activity was assayed by the method of Herbert (7). Units of catalase activity are expressed as the first order velocity constants (expressed in sec^{-1}) as obtained by that amount of catalase in the standard assays. All data represent the average of determinations on duplicates in which the variation did not exceed ± 5 per cent. Similarly, all experiments have been repeated at least once, and in most cases three separate experiments have yielded comparable results.

[5] A small amount of residual protein from the serum in the medium adheres to the glass and is not removed by washing with saline. It is therefore necessary to run blank tubes with no cells in complete medium in order to obtain the exact amount adherent to the glass. Lack of attention to this detail can result in inaccurately high protein values.

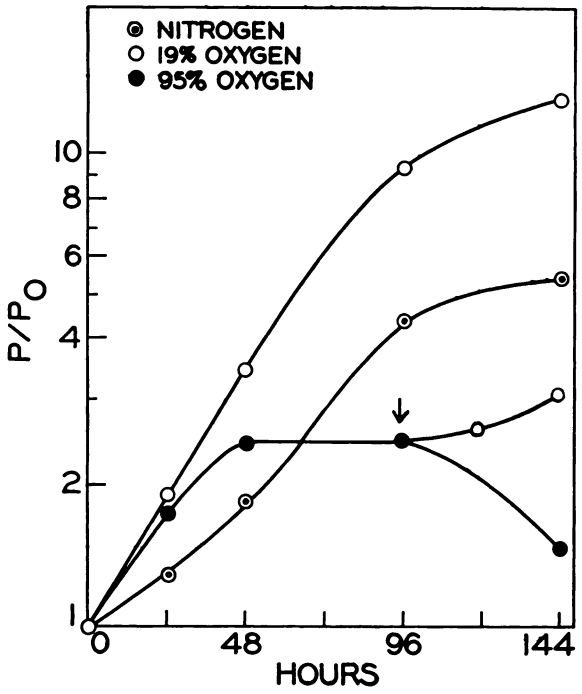

CHART 1.—HeLa growth kinetics in air, nitrogen, and oxygen. Cultures grown in test tubes as described under "Materials and Methods." 50,000 cells were used in the inoculum. Zero time cultures contained 68 μg. protein. Cultures were gassed with air, nitrogen, or oxygen, respectively. Growth is expressed as the ratio of protein present at any given time to that present at zero time. At the point indicated by the arrow the oxygen atmosphere was replaced by an air atmosphere in a portion of the cultures.

RESULTS

Inhibition of cell growth by oxygen.—Chart 1 compares the growth response of cells cultured under atmospheres of 0, 19, and 95 per cent oxygen, respectively. Under normal growth conditions (19 per cent oxygen) the cells grew and accumulated protein at a constant rate for a period of 4 days and then slowed as the medium became exhausted. With 95 per cent oxygen, however, the cells grew at a slightly decreased rate during the first 48 hours but then ceased growing abruptly. Striking optical changes attended the cessation of growth; the cells became very transparent, highly

extended, and difficult to delineate under the microscope. After 96 hours some cells began to become detached from the glass and were lost from the analysis. Reduction of the oxygen level at 48 hours to 19 per cent resulted in a resumption of growth after a 1-day lag; however, 5–10 per cent of the population at 120 hours consisted of abnormally large cells with a high incidence of aberrant tripolar and tetrapolar divisions. Apparently, these cells were capable of growth but sustained some damage in their mitotic processes. The

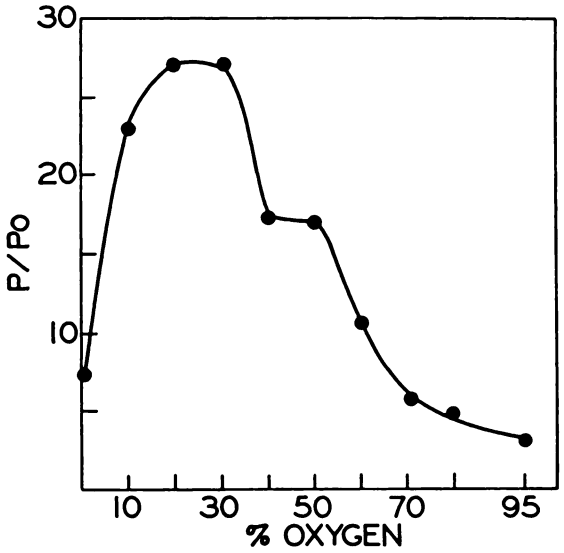

CHART 2.—Dependence of HeLa growth on oxygen tension. Culture conditions as under "Materials and Methods." 50,000 cells in 5 ml. BEHM were inoculated into 3-oz. prescription bottles after incubation for 24 hours to permit attachment. The experiment was initiated by gassing the flasks without changing the medium. At zero time the cultures contained 40 μg. protein. Data are expressed as the ratio of protein accumulated in a 4-day growth period to that present at zero time. Each point represents the average value of duplicate flasks. Variation seldom exceeded ± 5 per cent.

growth inhibition due to high oxygen tension could also be released after a 4-day exposure by reduction of the oxygen level. However, by 96 hours only a small fraction of the population remained viable (see also Chart 4).

When oxygen was omitted from the gas phase the cultures exhibited an initial lag but then accelerated to a near normal growth rate. The response was suggestive of an adaptive alteration to the anaerobic environment. Since the method of gassing did not remove the traces of oxygen dissolved in the medium it is not known whether or not HeLa cells proliferate in the complete absence of oxygen; it is, however, clear that these cells grow at nearly normal rates despite exceedingly low oxygen tensions.

In order to determine the concentration of oxygen which yielded half maximal growth, cultures of HeLa cells were grown for a period of 4 days under a range of oxygen tensions (Chart 2). In a number of such experiments oxygen tensions of 55–60 per cent decreased the accumulative growth to 50 per cent of maximum. The inhibition produced by high oxygen tension was not immediate but came on gradually to achieve maximal effectiveness at between 24 and 48 hours of exposure. Even with maximal oxygen toxicity the level of cell protein was doubled or tripled before growth ceased.

Effect of high oxygen levels on cell composition. —In Chart 3 the effect of a 95 per cent oxygen atmosphere is shown on the processes of cell divi-

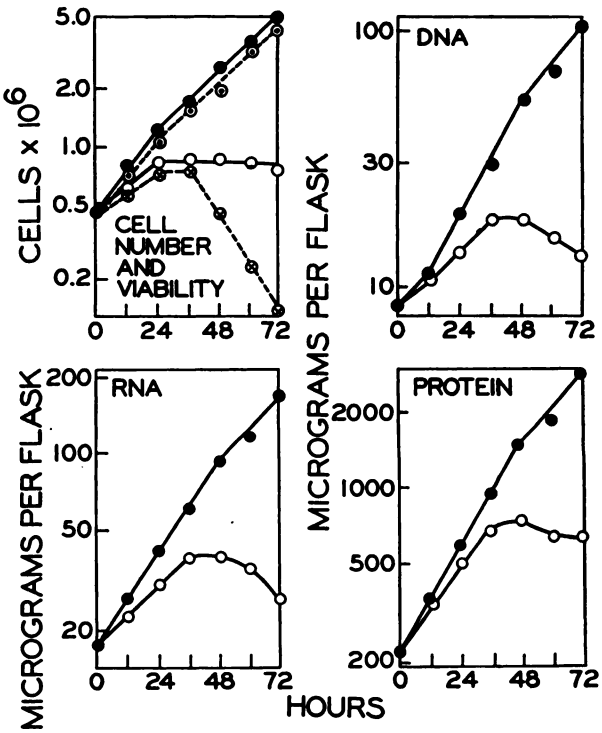

CHART 3.—Effect of high oxygen tension on cell division, viability, and macromolecular biosynthesis. 0.25 × 10^6 cells in 10 ml. BEHM were inoculated into 3-oz. prescription bottles, and, after 24 hours were allowed to permit attachment of the cells to the glass, the flasks were flushed with their respective gas mixtures for 15 seconds at a flow rate of about 3 l/min without changing the medium. Zero time is the time at which the gas mixtures were admitted to the glass-attached cultures. ●—— 5 per cent CO_2 in air; ○—— 5 per cent CO_2 in oxygen; ⊙ - - - viability in air; ⊗ - - - viability in oxygen.

sion, DNA, RNA, and protein synthesis. It is apparent that cell division is the most sensitive and is blocked first; DNA, RNA, and protein all continue to accumulate for an additional 12 hours.

When cells were tested for viability by the cloning technic of Puck and Marcus (14), it was observed that the viability declined at the rate of 75 per cent per generation time after the macromolecular syntheses were stopped (i.e., beyond 36 hours).

Effect of oxygen tension on glucose metabolism.—In Table 1 the influence of oxygen concentration on the metabolism of glucose is summarized. It can be seen that at all concentrations of oxygen a major portion of the glucose metabolized could be accounted for as lactic acid; at high oxygen tensions the response was similar to a completely anaerobic system. Only at oxygen tensions of 20–30 per cent do the alternative pathways of metabolism for glucose operate effectively. Calculating the specific rate of glucose utilization as the micrograms of glucose metabolized/hour/mg of cell protein (Chart 4) demonstrates a significant Pasteur effect at oxygen concentrations around 20 per cent; however, this effect decreased as the oxygen tension increased.

TABLE 1

INFLUENCE OF OXYGEN TENSION ON GLYCOLYSIS

PER CENT OXYGEN	96 HOURS			120 HOURS		
	G	L	L/G×100	G	L	L/G×100
0	4100	4100	100	5150	4950	98
10	4150	3500	85	5200	4950	70
20	3600	2650	71	4700	2560	55
30	4300	3100	72	5050	3120	62
40	4500	4000	89	5100	3590	71
50	4650	4350	94	5150	4450	86
60	4850	4750	98	5150	3820	74
70	4750	4800	101	5200	4540	87
80	4500	4600	102	5150	4650	90
95	3850	3900	102	5100	5150	101

G = μg. glucose used per culture. L = μg. lactic acid produced per culture. Data are derived from the experiment described in Chart 2. Each culture contained 5250 μg. of glucose.

From the data on glucose utilization and cell growth it can also be concluded that efficiency of using the energy derived from the glycolysis of sugar under high oxygen tensions must be very low, since, although the growth of the cultures was strikingly limited by high oxygen tensions, the metabolism of glucose proceeded at an even faster rate than under normal oxygen tensions. Even under anaerobic conditions where the yield of cell material was nearly normal, it can be calculated that less than 12 per cent of the phosphate bond energy generated from the glycolysis of the glucose would be needed to carry out the known high energy-requiring reactions involved in the synthesis of the protein, DNA, and RNA which accumulated in these cultures.

Attempts to counteract oxygen toxicity.—Since it had been reported previously by Lieberman and Ove (11) that catalase effectively reduced the toxicity of aerobic conditions on cells cultured in the absence of serum, varying levels of catalase were tested for protection in the present experiments. However, this material was found to be completely ineffective in our system, which contained serum (Table 2). On the other hand elevation of the serum concentration in the tissue culture medium did afford a slight protective effect (Table 3), but did not prevent the eventual lethality of 95 per cent oxygen.

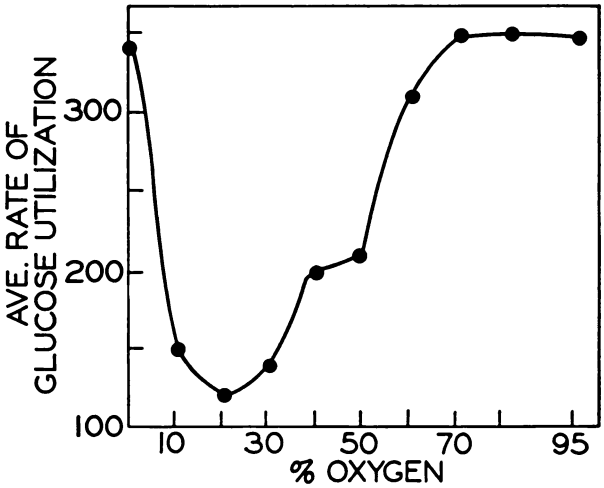

CHART 4.—Dependence of rate of glucose utilization on oxygen concentration. The rate data, expressed as μg. glucose used per hour per mg. protein, were calculated on the basis of glucose disappearance between zero and 96 hours by the formula, rate = 2300 ΔG log $(P/P_o)/96\ \Delta P$, which takes into account the steadily increasing amount of protein.[6] ΔG = micrograms glucose used per culture over the 96-hour period, ΔP = micrograms protein accumulated, and P/P_o = relative proliferation. This formula is not applicable to the case of 70, 80, and 95 per cent oxygen tension, since the growth rate is not constant but decelerating. In these cases the rates were estimated from glucose used between 48 and 96 hours, when little or no protein accumulated in these cultures.

In attempts to prevent oxygen toxicity with reducing agents, ascorbic acid, reduced glutathione, and thiosulfate were tried unsuccessfully; these agents were either inhibitory or inactive. Similarly, no beneficial effects were obtained with mer-

[6] Only the initial and final glucose concentrations and amounts of protein are determined. Since the amount of protein is increasing at an exponential rate, it is necessary to derive an expression relating the quantities measured to the "instantaneous rate" of glucose utilization. Such an expression may be derived as follows

If the rate of glucose utilization at any given instant is proportional to the amount of protein in the cells, this can be symbolized as (1) $-dG/dt = aP$, where $-dG/dt$ is the rate of glucose utilization, P is the weight of protein, and a is the

captoethanol (10^{-6}, 10^{-5}, 10^{-4}, or 10^{-3} M) or by yeast extract at 0.5, 0.05 or 0.005 per cent. Leucovorin, however, relieved oxygen inhibition about 15 per cent at 10^{-6} M, but higher concentrations (up to 10^{-4} M) were no more effective, and death invariably ensued. A mixture containing fumarate, succinate, malate, pyruvate, glycine, serine, aspartate, and ribose, all at a final concentration of 10^{-4} M, was also completely ineffective in protecting against oxygen poisoning.

TABLE 2

ATTEMPTS TO REVERSE OXYGEN INHIBITION WITH VARIOUS AGENTS

	Conc.	P/P_o
Oxygen control		2.5
Catalase	10^{-3} units/ml	2.5
Catalase	10^{-2} units/ml	1.9
Catalase	10^{-1} units/ml	1.7
Ascorbic acid	10^{-3} M	1.7
Ascorbic acid	10^{-4} M	2.2
Reduced glutathione	10^{-3} M	2.4
Reduced glutathione	10^{-4} M	2.5
Thiosulfate	10^{-3} M	1.4
Thiosulfate	10^{-4} M	2.3
Air control		12.8

Cultures grown in test tubes as described under "Materials and Methods." One ml. growth medium containing 19,000 cells was inoculated into test tubes and allowed 24 hours to become attached to glass. The medium was then replaced with 4.00 ml. of fresh medium containing the indicated materials at the appropriate concentration. All tubes except the air controls were then gassed with 5 per cent CO_2 in oxygen. Growth was terminated on the 6th day. P/P_o is the ratio of the protein found on the 6th day to that found on the day the experiment was initiated.

DISCUSSION

While the mechanism of oxygen toxicity remains unsolved, it seems indicated that some very fundamental process of the cell must be affected, since the synthesis of the polymers (i.e., DNA, RNA, and protein) was inhibited in a concerted manner as an initial response to high oxygen tension. In this connection it is interesting that these syntheses, though suppressed, were sustained until 36 hours (Chart 3), at which time the further accumulation of these polymers was blocked completely. At this point it was noted that the viability of the cells also began to decline, and this decline proceeded logarithmically during the ensuing 36 hours. The logarithmic nature of this decline suggests that oxygen promoted the alteration of a single factor which was primarily responsible for the loss of viability. Since the oxygen effects are exerted immediately on the polymer synthesis, it seems that the suggested alteration by oxygen of some factor needed for viability of the cells might also be initiated immediately and continued at a constant rate. In such a case extrapolation of the declining viability curve back to zero time would suggest that the starting cells contained approximately 10 times the amount of some potential "viability factor" as is needed to maintain the viable cell.

TABLE 3

EFFECT OF DIALYZED SERUM PROTEIN ON OXYGEN TOXICITY

Dialyzed serum concentration (%)	Growth (per cent of control)
5	6
10	11
20	11
40	18
60	20
80	21
95	26

Cultures grown in test tubes as described under "Materials and Methods." All the components of complete Eagle's medium were adjusted to the proper concentration in serum which had been previously dialyzed twice at 4° C., for 24 hours against 100 volumes of balanced salt solution from which glucose and sodium bicarbonate had been omitted. Dry glucose and bicarbonate were added to the proper concentration, the serum was filtered, and the other components were added as concentrates to their proper concentration. The protein increase (growth) of cultures under oxygen was compared with that of its control at a comparable serum concentration. Cultures grown under 95 per cent oxygen were compared with controls grown under air.

While only further experiments will demonstrate whether or not this situation applies, it is clear in any case that some very basic deficiency

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specific rate constant for glucose utilization. The amount of protein present at any given time in an exponentially growing culture can be expressed as (2) $P = P_o e^{\beta t}$, where P_o is the protein at zero time, t is the time of growth, and β is the constant for protein production. Equation (2) may also be expressed as

$$\beta = \frac{lnP/P_o}{t}.$$

Substituting for P in Equation (1) and integrating yields

$$G = \frac{a\,(P_o e^{\beta t} - P_o)}{\beta} = \frac{a\,(P - P_o)\,t}{lnP/P_o},$$

and the rate of glucose utilization, a, is

$$a = \frac{\Delta G 2{,}300 \log P/P_o}{t\Delta P}$$

where ΔG is glucose disappeared and ΔP is protein produced.

arises about 36 hours after exposure to high oxygen tensions which completely precludes further accumulative synthesis of DNA, RNA, and protein in the culture. This condition prevails, despite the fact that the cultures continue to glycolyze glucose at a very rapid rate and could therefore be expected to have considerable energy available for growth processes. Again, since a number of the glycolytic enzymes contain functional sulfhydryl groups it seems unlikely that the random oxidation of such groups in enzymes has been extensive. As to the actual molecular nature of oxygen toxicity Gerschman (6) has pointed out some similarities between oxygen poisoning and x-radiation and postulates a common mechanism based on the formation of oxidizing free radicals. The numerous aberrant mitoses observed following exposure of HeLa cells to high oxygen tension are in accord with such a concept but give no clues concerning the biochemistry of the phenomenon. On the other hand, the failure of the reducing agents to afford any protection against high oxygen tensions does not provide evidence for this concept.

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Cancer Res 1960;20:944-949.