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Effects Of And Recovery From High And Low Oxygen Tensions On A Strain Of Mouse Fibroblasts Cultivated In Vitro

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EFFECTS OF AND RECOVERY FROM HIGH AND LOW OXYGEN TENSIONS ON A STRAIN OF MOUSE FIBROBLASTS CULTIVATED IN VITRO

A Dissertation

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Doctor of Philosophy

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David Harold Shaw

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EFFECTS OF AND RECOVERY FROM HIGH AND LOW OXYGEN TENSIONS

ON A STRAIN OF MOUSE FIBROBLASTS CULTIVATED IN VITRO

Abstract of Dissertation

The cytotoxic effect of high, as well as low, oxygen tension on proliferation and metabolism of Low Line cells in culture is reversible, even after several days of exposure provided the cells are returned to 95% air + 5% CO_2. This suggests that the activity of certain mechanisms within the cells may have been altered or in other ways inhibited by the abnormal environments but are quite rapidly regenerated once the adverse conditions are removed.

The cells tolerate a low O_2 exposure for at least 20 days while continuous exposure to high O_2 atmosphere results in degeneration and death after 7-10 days. Glucose utilization, pyruvic and lactic acid production are all elevated in cultures exposed to either low or high O_2 tensions, although they are markedly higher in the latter condition. When cells so exposed are returned to an air + 5% CO_2 atmosphere, rate of glucose uptake, pyruvic and lactic acid formation soon approaches that found in control cultures.

Elevation in acid phosphatase and lactic dehydrogenase activities was also noted in cells subjected to either high or low O_2 tensions, with the elevation greater in the former condition. When previously exposed cells are returned to air + 5% CO_2, these activities soon approach those of the control cultures. Alkaline phosphatase was not detected in the cells under study.

DNA, RNA, and protein content of the cells per ml. of culture medium in which the cells had grown follows approximately the same pattern as that of the results obtained for proliferation under these conditions. That is, there is a general decrease in the amounts of DNA, RNA, and protein per ml. in cells exposed to either high or low O_2 with the greatest decrease occurring in those subjected to high O_2. Cells which had been under these conditions for several days and then transferred to air + 5% CO_2 demonstrated a sharp rise in DNA, RNA, and protein per ml. which then approached that found in the controls. When expressed on a per cell basis this same general pattern is noted with several exceptions. The RNA per cell in cultures under low O_2 tension is higher than in control cells but this returns to control levels once the low O_2 atmosphere is replaced with air + 5% CO_2. The amount of protein per cell under either high or low O_2 tensions is somewhat higher than in control cells. These levels return to control levels once the adverse conditions are removed.

Cytochrome oxidase activity appears not to be affected by either high or low O_2 environments. Succinic dehydrogenase is inhibited by high O_2 tension but does not seem to be affected by low O_2 tensions. The activity of the latter enzyme reappears once the high O_2 is removed and replaced with air + 5% CO_2. Both high or low O_2 tensions initiate cellular lipid accumulation which disappears once the toxic conditions are removed.

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ABSTRACT

EFFECTS OF AND RECOVERY FROM HIGH AND LOW OXYGEN TENSIONS ON A STRAIN OF MOUSE FIBROBLASTS CULTIVATED IN VITRO

The cytotoxic effect of high, as well as low, oxygen tension on proliferation and metabolism of Low Line cells in culture is reversible, even after several days of exposure, provided the cells are returned to 95% air + 5% CO_2. This suggests that the activity of certain mechanisms within the cells may have been altered or in other ways inhibited by the abnormal environments but are quite rapidly regenerated once the adverse conditions are removed.

The cells tolerate a low O_2 exposure for at least 20 days while continuous exposure to high O_2 atmosphere results in degeneration and death after 7-10 days. Glucose utilization, pyruvic and lactic acid production are all elevated in cultures exposed to either low or high O_2 tensions, although they are markedly higher in the latter condition. When cells so exposed are returned to an air + 5% CO_2 atmosphere, rate of glucose uptake, pyruvic and lactic acid formation soon approaches that found in control cultures.

Elevation in acid phosphatase and lactic dehydrogenase activities was also noted in cells subjected to either high or low O_2 tensions, with the elevation greater in the former condition. When previously exposed cells are returned to air + 5% CO_2, these activities soon approach those of the control cultures. Alkaline phosphatase was not detected in the cells under study.

DNA, RNA, and protein content of the cells per ml. of culture medium in which the cells had grown follows approximately the same pattern as that of the results obtained for proliferation under these conditions. That is, there is a general decrease in the amounts of DNA, RNA, and protein per ml. in cells exposed to either high or low O_2 with the greatest decrease occurring in those subjected to high O_2. Cells which had been under these conditions for several days and then transferred to air + 5% CO_2 demonstrated a sharp rise in DNA, RNA, and protein per ml. which then approached that found in the controls. When expressed on a per cell basis this same general pattern is noted with several exceptions. The RNA per cell in cultures under low O_2 tension is higher than in control cells but this returns to control levels once the low O_2 atmosphere is replaced with air + 5% CO_2. The amount of protein per cell under either high or low O_2 tensions is somewhat higher than in control cells. These levels return to control levels once

the adverse conditions are removed.

Cytochrome oxidase activity appears not to be affected by either high or low O_2 environments. Succinic dehydrogenase is inhibited by high O_2 tension but does not seem to be affected by low O_2 tensions. The activity of the latter enzyme reappears once the high O_2 is removed and replaced with air + 5% CO_2. Both high or low O_2 tensions initiate cellular lipid accumulation which disappears once the toxic conditions are removed.

INTRODUCTION

INTRODUCTION

The mind of man demands an explanation for the phenomena and mysteries of nature. One of the most challenging, but difficult, is the study and understanding of the mystery of life. The mystery of life is regarded by many as beyond human understanding, yet it has been slowly revealing itself in the light of much scientific investigation. Although still unsolved, many procedures, techniques, and methods have been followed in an attempt to broaden our knowledge in this direction. One of the most useful tools in this endeavor are the methods, procedures and techniques employed in the cultivation of cells and tissues in vitro.

Tissue culture is an area of scientific endeavor which could develop only after the cell was recognized as the structural and functional unit of all living matter. This cell doctrine was widely elucidated and popularized by Schleiden and Schwann during the first part of the 19th century, although many other investigators previous to this time deserve considerable credit for collecting evidence in its favor.

The idea of tissue culture became enhanced through the works and concepts of the famous French physiologist, Claude Bernard. It was Bernard (1878) who first realized that the cell environment is partly the product of cell metabolism, which reacts in turn upon the cells and tissues themselves,

thereby to some extent controlling and regulating their activity.

The beginnings of tissue culture is generally dated with the investigations of Ross G. Harrison (1907) who first cultivated nervous tissue from frog embryos. It should be noted though, that many earlier attempts had been made on the possibility of growing tissue outside the organism. William Roux in 1885 isolated the medullary plate of a chick embryo in warm saline solution and kept it alive for several days. Ljungren (1898) transplanted bits of living human skin into ascitic fluid as nutrient medium and maintained them for weeks. Leo Loeb (1902) planted small fragments of guinea-pig epidermis in blocks of agar and clotted plasma which he placed for incubation in subcutaneous pockets of another animal. Jolly (1903), using hanging drop preparations, was able to carry out an extensive observation on amphibian leucocytes kept in serum for 30 days. This was the first detailed observation on cell survival and maintenance in vitro.

These investigations along with others inspired further attempts to maintain and cultivate living tissue outside the body. Yet it remained for Harrison to devise a simple technique that would permit explantable parts to continue to grow and develop outside of the organism. He cultivated the neuroblast of the frog embryo in clotted frog

lymph and observed the growth of the fibrillae from the central body. His cultures not only showed evidence of growth, but the cells could be kept in good condition for several weeks, and could be observed from day to day. This, then was the advent of tissue culture.

The original procedure of Harrison, although monumentous in its achievement, did not allow the tissues to be maintained under constant conditions and their properties to be analyzed. Other techniques had to be devised for these procedures to advance.

Scientists are indebted to Burrows (1912) for his early interest and enthusiasm in continuing and elaborating on the work of Harrison, and to Carrell (1914-1931) for the extension and refinement of the basic techniques of tissue culture. Carrell quickly saw the fulfillment of the ideas laid down by Claude Bernard by utilizing these methods. Since then, many researchers have devised and refined numerous techniques and procedures employed in tissue culture today and have brought it from a much criticized research tool to one of great prominence among the scientific community.

Cultivation of tissue cells <u>in vitro</u> has been used for many purposes in Experimental Physiology, Pharmacology, Cytology, Biochemistry, etc. The procedure has provided scientists the means with which to study a multitude of

phenomena, such as the nature of cytoplasmic organelles; the process of cell division in all its phases; the nutritional requirements of various cell types, and the nature of these requirements in a chemically defined nutritive medium; the nature of cell movement; the difference and similarities between tumor and normal cells of similar origin.

Of all the vast areas of possible studies unveiled by means of cells cultivated in vitro, one of the most important and complex undertakings has been that corcerned with cellular respiration.

Except for organisms that are especially adapted to live under anaerobic conditions, all animals and plants need oxygen for the production of energy and maintenance of life. Yet oxygen at much higher or lower concentrations than that present in air is toxic to life. This paradox is one of the most fascinating problems of Biology. Gilbert (1964) expresses the idea that the development of resistance to oxygen toxicity probably played a major role in evolution. For organisms and species to survive, it became necessary for them to acquire not only the enzymes catalyzing oxidative and synthetic reactions but also protective mechanisms against excessive oxidation of cellular constituents by oxygen. The understanding of these mechansims is still imperfectly understood but is of fundamental importance to the problems of oxygen toxicity.

The idea that oxygen toxicity involved inhibition of essential cellular reactions goes back many years. Bean (1941) established that oxygen was capable of inactivating a number of enzymes and that respiration of tissue homogenates and slices was markedly depressed during hyperbaric oxygenation. Stadie and Haugaard (1945) and Dickens (1946) published reports that greatly extended the earlier observations, and these investigators concluded that although some enzymes were resistant to oxygen toxicity, many others were easily inhibited by oxygen at high pressure. Among the enzymes they reported as particularly susceptible to oxygen toxicity were those containing essential sulfhydryl groups.

The enzyme, Succinic dehydrogenase, a flavoprotein with free sulfhydryl groups, has been shown to be inactivated by oxygen in studies employing rat liver, kidney, and brain homogenates (Stadie et al., 1945). This is one of the essential enzymes in the citric acid cycle.

Studies on the effects of oxygen on cells in culture have been numerous. Tissue culture techniques are advantageous in that oxygen tension can be carefully controlled and intact cells can be studied under conditions at which any changes in the rate of diffusion of oxygen do not influence the results.

To the aerobic cell, oxygen is the most important component of the air. Few cells will survive for very long

periods under complete anaerobic conditions. However, certain cells may survive and even multiply to a limited extent in the complete absence of oxygen if they are freely supplied with glucose. Therefore, such cells usually display a high anaerobic glycolytic capacity; energy being derived from glycolysis with lactic acid accumulation (Willmer, 1954).

Much work has been published on the effect of varied oxygen concentrations on cellular proliferation. Burrows (1917) using tissue fragments of heart muscle and skin cells of chick embryos cultured in chick plasma found that cells grow actively in pure oxygen, but this growth is no greater than that in an atmosphere containing 9 or 10% oxygen. Little growth was seen when the partial pressure of oxygen was as low as 45.6 mm. Hg (6.6% oxygen). King (1933) found that incubation of chick embryo tissue under oil (therefore without oxygen) caused serious damage to the tissues as expressed by an increase in the lag period and a decrease in growth. Finally, there is a complete lack of growth. In Amoeba proteus, both nuclear growth and fission can be retarded by either a marked increase or decrease in the oxygen tension (Chalkley and Voegtlin, 1940); exposure to 100% oxygen for 24 hours is found to irreversibly retard nuclear growth and fission in these cells. Bean (1941) reported that oxygen at just over 900 mm. Hg pressure completely inhibits growth of pneumococci, and at 4600 mm.

Hg kills these organisms. Chilomonas and Tetrahymena were found to live and reproduce in solutions exposed to a wide range of oxygen concentrations, but Chilomonas is killed at oxygen tensions of 600 mm. Hg or greater and possibly at somewhat lower concentrations (Pace and Ireland, 1945).

In more recent studies, Bonting and Jones (1956) found that in 15-day cultures of chick embryonic lung cells and intestinal cells cultivated in an atmosphere of 100% nitrogen, growth was almost normal for the 8 days of the experiment, while those cultures exposed to 100% oxygen became inactive after 4 to 6 days. Cooper, Burt, and Wilson (1958) observed that 25 to 30% oxygen is cytocidal to rabbit kidney cells in suspension.

Studies on the effects of various oxygen tensions on established cell lines have also been reported. Ruechert and Mueller (1960) showed that the growth of human cervical carcinoma cells is retarded by concentrations of oxygen greater than 30%. At 95% oxygen, growth is almost completely inhibited and the rates of synthesis of DNA, RNA, and protein is severely retarded. Brosemer and Rutter (1961), with a strain of mammalian fibroblast (AH), observed no growth and progressive cellular degeneration in cells exposed to 95% oxygen. DNA synthesis was also greatly diminished in these cells. Fisher (1960) studied the growth of L-Strain fibroblasts and noted that, when the concentration of oxygen in the gas phase was increased

above 5%, there was a delay in the onset of growth that was longer, the higher the concentration of oxygen; with 50% oxygen the delay was as long as 24 hours. Siegel et al., (1963) found that the growth of HeLa cells was progressively inhibited as the concentration of oxygen was increased above 30%. At the elevated tensions of oxygen the cells contained lipid droplets, not seen in cells grown in air.

Pace et al., (1962) studied the effects of oxygen tension on four established cell lines consisting of: (1) subcutaneous mouse fibroblast cells, NCTC Clone #929, Strain L (Sanford et al., 1948), (2) mouse liver cells, NCTC Clone #1469 (Evans et al., 1952), (3) human cervical carcinoma cells, HeLa (Gey et al., 1952) and (4) human skin epithelial cells, NCTC Clone #1769 (Perry et al., 1957). Depending upon the cell line investigated, high oxygen tensions (95-100%) slow growth and death occurs in 8-14 days. Similarly, the growth of cells in low oxygen tension (0%) is retarded; however, cell proliferation continues and the cells do not die within the 14 days of the experiment. Conversely, Hudspeth et al., (1950) observed that there is no outgrowth of chick embryonic spinal cord in 0% oxygen, but in 96% oxygen abundant outgrowth of fibers occurs.

Clark (1964) found that adult mouse fibroblasts do not survive at any oxygen tension of less than 1%, but

above 3%, growth is not affected.

Goldblatt and Cameron (1953) reported that mouse fibroblasts cultivated *in vitro* undergo considerable morphological changes and acquire malignant characteristics when exposed intermittently to deficient oxygen concentrations over long periods, whereas in control cultures, no such changes appear. Later Sanford (1965) and Sanford and Parshad (1968) extensively tested Goldblatt and Cameron's postulated on several strains of cells and concluded that anaerobic conditions have no influence on time or manifestation of malignant conversion, although both high and low oxygen concentrations inhibit cell proliferation. They also suggested that the type of culture medium used may alter the cell's response to anaerobiosis.

Carbohydrate metabolism has also been extensively investigated on cultured cells. Cori and Cori (1925) reported that lactic acid content of rat malignant tumors *in vitro* ranges from 0.01% and intravenous administration of glucose increased the lactate concentration of the malignant but not the benign tumors. Likewise, Warburg (1930) found that cancer tissue has a high rate of lactic acid production under aerobic as well as anaerobic conditions.

Wilson *et al.*, (1942), using chick embryonic tissues, reported that glucose utilization increases with increase

of glucose in the medium and that lactic acid forms in the presence or absence of glucose, although its formation increases with increase in glucose utilization. Westphal et al., (1955) using a relatively high concentration of glucose (240 mg.%) in the medium, observed lactic acid production of HeLa cells to be 25% of the glucose used. Bailey et al., (1958), working with a strain of mouse lymphoblast, found lactic acid production to occur at a rate of 70% lactic acid to 100% glucose utilized.

Broda et al., (1961) using chick mesenchyme tissue observed that as the number of cells increases, glycolysis per cell decreases but respiration per cell increases. Rucker et al., (1963) using chick mesenchyme cells and HeLa cells found that uptake of glucose per cell per hour during glycolysis decreases with an increasing number of cells. On the other hand, respiration increases with an increase in cell number and in time. However, Fulton (1956) using HeLa cells found no correlation on increase in cell number with glucose utilization.

Although much has been published on general carbohydrate metabolism, relatively little attention has been directed to the actual biochemical processes altered or disrupted as a result of high or low oxygen toxicity. Much has been learned about cellular reactivity to various oxygen tensions, but much more must be done before we can

begin to understand the basic cellular mechanisms involved. Little, if any, attention has been given to the process of recovery of cells following exposure to high oxygen tensions or complete oxygen lack. Because of this and because of the conflicting reports regarding the effects of high oxygen tension on glycolytic and respiratory metabolism the following investigation was initiated.

The aim of this project, therefore, was to (1) observe the effects of high oxygen tensions as well as anaerobiosis on an established cell line in order to compare the results obtained with a line of low malignant potential with some results reported earlier, (2) determine if recovery of cells propagated for some time under high or low oxygen tension is possible, and (3) ascertain via various biochemical analyses intracellular and extracellular changes that may occur when cells are subjected to different O_2 tensions, in order to obtain a better understanding of the processes involved.

MATERIALS AND METHODS

Materials and Methods

Origin and Nature of the Cell Strain Used

The cells used in these studies were the so-called Low Line, developed by Sanford, Likely, and Earle (1954), and classified as NCTC Clone #1742 (High Line) were both derived originally from one and the same non-malignant cell, isolated from a 97-day old parent culture of normal subcutaneous adipose connective tissue originally obtained from a C3H/He mouse. Before cloning these cells were apparently non-malignant; no tumors developed when they were injected into the same strain of mice from which they had been obtained. Injections of cells of the High Line and Low Line cultures first produced tumors in normal C3H/He mice after the 12th and 19th transplant generations, respectively. After 1-1/2 years, the percentage production of sarcomas was 63 per cent for the High Line and 0 per cent for the Low Line cells; after 3 years, it was 97 percent and 1 percent, respectively.

Cultivation of the Cells

The cells were cultivated in pyrex glass T-flasks (Earle, 1951) and maintained at 37.5°C in a National Appliance Company incubator (model #3376). T-60 flasks were used for stock cultures, and either T-15 or T-60 flasks were used for experimental cultures. Silicone stoppers

were employed for all the flasks; they contain a minimum of toxic substances that could dissolve in the medium. T-15 flasks have fairly good optical properties, are constructed so as to make the entrance of bacteria and other contaminants difficult, and are easily manipulated and stored.

All glassware was rinsed in running tap water immediately after use and then placed in a detergent solution for at least 30 minutes. After that, it was removed and rinsed 3 or 4 times in running tap water, and immersed for several hours in chromic acid. The chromic acid was then removed by rinsing the glassware at least 15 times with tap water and 3 times with distilled water. The glassware was then placed in an oven for drying at 250°C, after which it was ready for wrapping. With the exception of rather large pieces, the glassware was wrapped in toweling and paper and all pieces were sterilized in an Amsco 8816M autoclave at 250°F for 30 minutes, then dried for 15 minutes.

NCTC #109[1] (Evans, et al., 1956) supplemented with 10% newborn calf serum[2] served as nutrient medium. This medium was originally developed for long-term cultivation of strain L cells, but it has also proven to be effective

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[1]Obtained in dry form from Grand Island Biological Company, Berkeley, California.

[2]Obtained from Hyland Laboratories, Inc., Los Angeles, California.

in the cultivation of other cell strains as well, when supplemented with 10% serum. It, along with the serum has been used for cultivation for all cell lines in this laboratory for a number of years. Care was taken to ensure reasonable constancy of the serum for each experimental run by freezing large volumes at a time from the same batch and drawing from this frozen pool as needed. After its preparation, medium 109 plus 10% newborn calf serum was sterilized by passage through a sterile Millipore membrane filter with an average porosity of 0.22 microns and filtered into sterile 500 ml bottles for storage. The medium was stored at 4°C, and each time, preceding its use, it was refiltered through a sterile 0.22 micron Millipore membrane filter.

Cell transfer, medium change and gassing procedures were all carried out under a Lab Con Co tissue culture hood (Fig. 1). This was sterilized with a UV light for 20 minutes before use which proved to be very efficient as little trouble was encountered with contaminants.

Replicate Culture Technique

The replicate techniques used were the same as those devised by Pace and Aftonomos (1957). Cells were suspended in the stock cultures by scraping with a platinum wire. The suspension was shaken gently to separate the individual

FIGURE 1

Lab Con Co Tissue Culture Hood

cells. It was then drawn into a replicator (Fig. 2) from which 2 ml. aliquots were dispensed into several T-15 flasks for counting following which the cell density was adjusted to the desired level by dilution. A previously designated number of replicate cultures were then set up. Two ml. aliquots of the suspension were used for the T-15 flasks and 8 ml. for the T-60 flasks.

Forty-eight hours after replication, the medium in each T-15 flask was removed and replaced with 2 ml. of fresh medium dispensed from a duplicator (Fig. 3). Likewise when T-60 flasks were used, 8 ml. of fresh medium was added after the spent medium was removed. This procedure was repeated every 48 hours throughout the duration of the experiment.

Gassing Procedure

The various gas concentrations and rates of flow were obtained by means of flowmeters (Fig. 4). The procedure was similar to that described by Pace et al., (1962) with some modification. For example the gas mixtures were not humidified and the gas delivery pipette was surrounded by a large glass bell which gives better protection against possible bacterial contamination. The diameter of the bell was about twice as large as the body width of a T-15 flask and the glass tubing could be inserted approximately 2/3 of the way into the flask. Each T-15 flask was gassed for 15 seconds at a rate of 300 ml. per minute so that the gas in

FIGURE 2

Replicator

FIGURE 3

Duplicator

FIGURE 4

Gas Flow Meters and Mixing Chamber

each flask was replaced about 5 times. When T-60 flasks were used experimentally, the gassing time was increased to 30 seconds.

Measurement of Growth

a. Electronic Counter

Growth, by increase in cell numbers, was established by means of a Coulter Counter (model B), an electronic device which ascertains the number and size of particles suspended in an electrolytic medium. The suspension of particles to be counted flows through a small aperture (100 μ), essentially one particle at a time. Each particle displaces electrolyte as it passes through the aperture, momentarily changing the resistance between the electrodes and producing a voltage pulse of magnitude proportional to particle volume. The resultant series of impulses is electronically amplified, scaled, and counted.

The cells were brought into suspension by gentle agitation of the flask after first incubating the cells for one-half hour in an isotonic, calcium-and magnesium-free "counting solution" containing Darvan, a dispersing agent, and Versene, a chelating agent.

b. Chemically

Growth was also measured by assaying for the total cellular protein, Deoxyribonucleic Acid (DNA), and Ribonucleic Acid (RNA). At the beginning of each experiment

and 24 hours after each medium change, the medium in the flask was replaced with a warm calcium-and magnesium-free balanced salt solution (BSS) containing 0.25% trypsin. This proteolytic enzyme causes the cells to detach from the surface of the flask. The resulting suspension was decanted into a 15 ml. conical centrifuge tube and centrifuged at 2000 rpm for 15 min. at 4°C in a Sorvall ultracentrifuge (model RC-2). The resultant cell pellet was subjected to extraction procedures as described by Merchant et al., (1964). This procedure is presented in Table 1.

Determination of Ribonucleic Acid (RNA) involves measurement of the pentose content by the orcinol reaction (Mejbaum, 1939). Deoxyribonucleic Acid (DNA) was ascertained by means of the method described by Burton (1956) which depends upon the reaction of diphenylamine with deoxyribose. Protein content was estimated by means of the Folin-Ciocalteau phenol reagent (Lowry et al., 1951). This method is based on the color reaction of the aromatic amino acids, tyrosine and tryptophane, with the Folin-Ciocalteau reagent.

Chemical Analyses of the Medium

Colorimetric analyses were made on the medium in which the cells were growing for glucose uptake and lactic acid and pyruvic acid production.

Glucose uptake was ascertained 24 hours after fresh

TABLE I

FLOW SHEET FOR THE SEPARATION OF RNA, DNA AND PROTEIN IN MAMMALIAN CELLS CULTURES *IN VITRO*.

Cell Pellet (0.5 to 2.0 x 10^6 cells)

1.0 ml 0.5N $HClO_4$
Allow to extract at 4°C for 30 min.
Centrifuge 4°C, 2000 RPM

Acid-soluble phosphate solution ← Supernatant

Residue
Wash 1x in absolute C_2H_5OH containing 0.2 N potassium acetate
Centrifuge at 2000 RPM at 4°C

Supernatant

Residue
Extract 2x with 1 ml portions of 3:1 C_2H_5OH-Ether mixture at 50°C for 30 min.
Centrifuge at 2000 RPM 4°C for 15 min.

Phospholipid fraction ← Combined Supernatants

Residue (lipid-free)
Resuspend in 1.0 ml 0.1N KOH
16-18 hrs. at 37°C.

Solubilized cell material

Add 0.034 ml 6N HCl and 1.0 ml 0.5N $HClO_4$
Allow precipitate to form at 4°C for 30 min.
Centrifuge at 2000 RPM 4°C for 15 min.

RNA
Mejbaum orcinol reaction ← Supernate

Residue - (DNA & Protein)
Resuspend in 1.0 ml 0.5N $HClO_4$
Heat 90°C for 15 min.
Cool and centrifuge at 2000 RPM 4° C for 15 min.

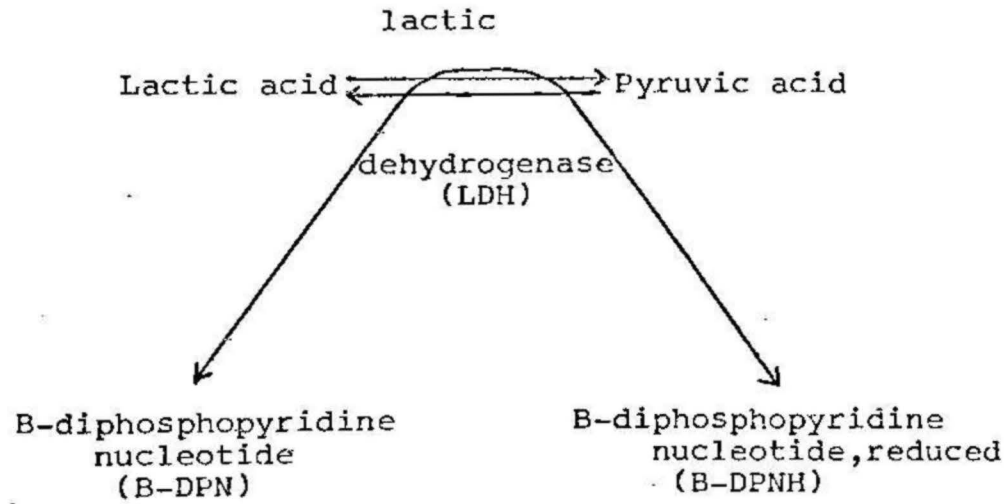
DNA
Burton modification diphenylamine reaction ← Supernate

Residue - (Protein)
Resuspend in 2.0 ml 1N NaOH
(may require overnight incubation at room temperature to put protein into solution).

Protein
Lowry modification Folin-Ciocalteau reaction ← Protein Solution

medium was added to the flask. The method used was described by Hyvarinen and Nikkili (1962). Although not entirely specific for glucose, the O-toluidene method was chosen since it is accurate, rapid and quite easy to manipulate. This method may also be used to determine galactose and mannose, but since these sugars are not present in the medium, they are of little or no consequence. Reliability of this method was checked by comparing it to the enzymatic glucose analysis (Keston, 1956); they checked within 0-4 mg.%.

Lactic acid and pyruvic acid were also ascertained 24 hours after replacement of the medium using the enzymatic methods.[3] The reaction involved is as follows:



[3]Lactic acid determination kits were obtained from Calbiochem Corp., Los Angeles, California. They contain glycine hydrazine buffer, lactic dehydrogenase, and DPN. Pyruvic acid kits were obtained from Sigma Chemical Co., St. Louis, Mo. They contain phosphate buffer, LDH, and DPNH.

The enzymatic method for lactic acid (Scholz, et al., 1959) involves measurement of the amount of DPN which is converted to DPNH in the presence of excess LDH. In the enzymatic determination of pyruvic acid (Sigma Tech. Bulletin No. 725) the amount of DPNH which is converted to DPN is measured. Both were measured at 340 mu be means of a Hitachi spectrophotometer (model #101).

Chemical Analyses of the Cells

Besides protein, DNA and RNA determinations on the cells, acid and alkaline phosphatase as well as lactic dehydrogenase (LDH) activity was measured colormetrically. Since more cells were needed in order to get within the sensitivity range of the procedures employed, the large T-60 flasks were used for culturing. At the beginning of the experiment and 24 hours after each medium change, acid and alkaline phosphatase and LDH activity was measured. The medium in the flask was replaced with warm 0.25% trypsin solution. The cells detached and the resultant suspension was decanted into 15 ml. conical centrifuge tubes. The culture flasks were rinsed with balanced salt solution (BSS) to remove any traces of the cells that still remained in the flask. The suspension was centrifuged for 15 minutes at 2000 rpm. The resultant cell pellet was washed three times with BSS and recentrifuged after each washing. After the third washing, the BSS was removed and

extraction medium was added to give a total volume of 2 ml. The extraction medium was prepared according to Shonk and Boxer (1964). It contained 0.15M KCl; 0.05M $KHCO_3$; and 0.006M Na_2 EDTA. The cell pellet was suspended in this extraction medium and the resultant suspension was homogenized for two minutes in the cold in a motor driven previously chilled 10 ml. Potter-Elvejhem homogenizer. One portion of the crude homogenate was used for measuring alkaline phosphatase activity; another portion for estimating acid phosphatase activity; a third portion was used to ascertain LDH activity. Samples of the homogenate were also frozen and later assayed for protein content according to the method of Lowry et al., (1951). Phosphatase and LDH specific activity is reported per mg of total cell protein extracted.

The phosphatases were determined by an adaptation of the method of Bessey, Lowry and Brock (1946), which depends on the enzymatic hydrolysis of p-nitrophenylphosphate. Appropriate aliquots of cell homogenate were incubated with buffered substrate for 30 minutes at 37°C. The reaction was stopped by the addition of alkali and the p-nitrophenol measured colorimetrically at 410 mμ. Acid phosphatase activity was measured in 0.05 M citrate buffer at pH 4.8 and alkaline phosphatase activity in 0.05 M glycine buffer at pH 10.5.

LDH activity was ascertained by the method of Berger

and Broida (1967); it catalyses the following reaction:

$$\text{Pyruvic Acid} + \text{B-DPNH} \xrightleftharpoons{\text{LDH}} \text{Lactic Acid} + \text{B-DPN}$$

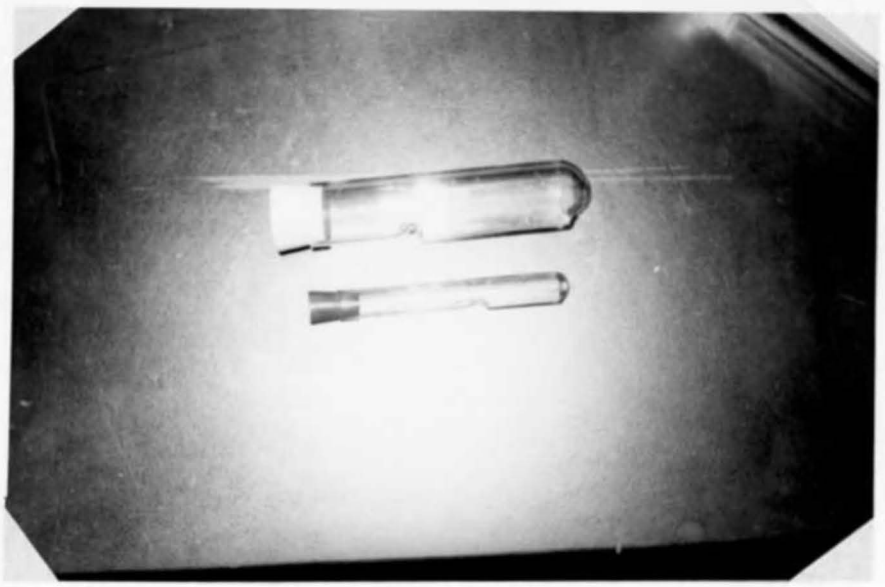
The speed of rate is proportional to the amount of LDH. Pyruvic acid reacts with 2,4-Dinitrophenylhydrazine to form an intensely colored "Hydrazone" which has a high optical density (O.D.) and is measured colorimetrically at 460 mμ. Lactic Acid, B-DPN, and B-DPNH do not contribute a significant O.D. at this wave length. Therefore, by starting with a standardized Pyruvate Substrate (Sigma Chem. Co.) which always yields the same hydrazone O.D., one can accurately measure the varying O.D.'s resulting from the conversion of part of the pyruvate to lactic acid due to the LDH activity. The amount of pyruvic acid remaining after incubation is inversely proportional to the amount of LDH present in the sample.

Histochemical Procedures

At the beginning of each experiment and 24 hours after each medium change, cells were stained for succinic dehydrogenase, cytochrome oxidase, and neutral fats. For this, cells were grown in Leighton tubes (Fig. 5) on 9mm x 22mm acid washed no. 1 coverslips. At appropriate time intervals the coverslip was removed and the staining procedure initiated.

Succinic dehydrogenase was demonstrated using the

FIGURE 5



Leighton Tubes

method of Seligman and Rutenburg (1951). The coverslips were taken from the Leighton tubes and incubated for 2 hours in an aqueous solution containing equal volumes of 0.1 M phosphate buffer (pH 7.6), 0.2 M sodium succinate, and Ditetrazolium chloride (1.0 mg/ml). The coverslips were then washed in saline (0.85%), the attached cells were hardened in 10% formalin and mounted in glycerogel. Succinic dehydrogenase causes the pale Ditetrazaolium chloride to be reduced either to a blue, water insoluble pigment (Diformazan), or a reddish-purple water insoluble pigment (Monoformazan) depending on the amount of Ditetrazolium chloride reagent used and the enzyme activity. The Succinic dehydrogenase activity is demonstrated by the granular deposit of this blue or reddish-purple pigment intracellularly.

Cytochrome oxidase activity was demonstrated by the technique of Burstone (1961). The coverslips were incubated in a solution of p-aminodiphenylamine plus a quinoline derivitive in tris buffer (pH 7.4) for 15 minutes or longer, after which they were fixed in 10% Cobaltous acetate in 10% formalin for approximately 1 hour. They were washed and mounted in glycerogel. A blue color forms at the site of enzyme activity.

The presence of neutral fats was demonstrated by fixing and staining the cells grown on coverslips with Oil-Red-O. A pink to bright red color is assumed by the fats,

depending on the length and procedure of staining.

Experimental Procedure

Usually, 110-130 replicate cultures containing 2 ml. of cell suspension per T-15 flask were set up for each experiment. Each culture contained from 1.2×10^5 to 1.6×10^5 cells per ml. In those experiments employing T-60 flasks, usually 40-50 replicate cultures containing 8 ml. of cell suspension per flask were used. The cells were allowed to "adjust" and attach for 48 hours before gassing was begun.

At the end of 48 hours cell counts were made on three flasks selected at random. The medium was then replaced with fresh medium and the cultures flushed with the desired gas mixture. Only about 30 seconds elapsed between the time the medium in a flask was changed and the flask gassed. These procedures were repeated following each medium change (every 48 hours).

Twenty-four hours after the first medium change, cell counts and chemical and histochemical analyses of the medium and cells were made. The cells in three T-15 flasks from each of the experimental groups were counted. Determinations for glucose, lactic acid and pyruvic acid were made on pooled media from the 3 flasks. Two T-15 flasks from each experimental group were used for protein, DNA, and RNA extraction procedures. Two T-60 flasks from each

experimental group were used for the acid and alkaline phosphatase and LDH activity determinations.

Some time later, those cells which were propagated in an adverse environment were removed and put into one of the other mixtures. The time of this transfer was determined by microscopic examination of the cells correlated with the growth curve.

RESULTS

RESULTS

The initial part of this project was concerned with the effects of high and low O_2 tensions on cellular growth, glucose utilization, and lactic acid production. These were initiated in order to confirm in Low Line cells the cytotoxic effects of these adverse environments as reported in other cell lines. If such effects were noted then the second phase was to ascertain whether or not these effects could be reversed by changing the environment to a more favorable one at a point when the cytotoxicity was clearly evident.

The results of these experiments on the cytotoxic effects of the two experimental conditions (Figs. 6-11) confirm those reported in previous investigations for other cell lines (Bonting and Jones, 1956; Rueckert and Mueller, 1960; Brosemer and Rutter, 1961; Pace et al., 1962; Clark, 1964.)

Within a week following exposure to high O_2 tensions, the cells are damaged to the extent that they begin to die and disintegrate. Their metabolism is changed to a more glycolytic type and growth ceases after 5 days.

Cells exposed to 95% O_2 attain a much lower growth maximum than those in control cultures. Proliferation is slight, in fact the growth curve might better be termed a "death curve". Maximum numbers are attained on about the

FIGURE 6. Compilation of 3 experiments showing growth of Low Line cells under "normal" conditions (95% air plus 5% CO_2) and in 95% O_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter condition to air or 95% N_2 plus 5% CO_2. Arrow to the left indicates the time the cells were exposed to 95% O_2; the other, the time of the gas changeover as indicated.

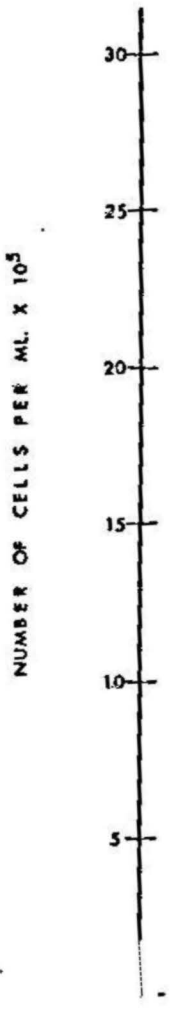
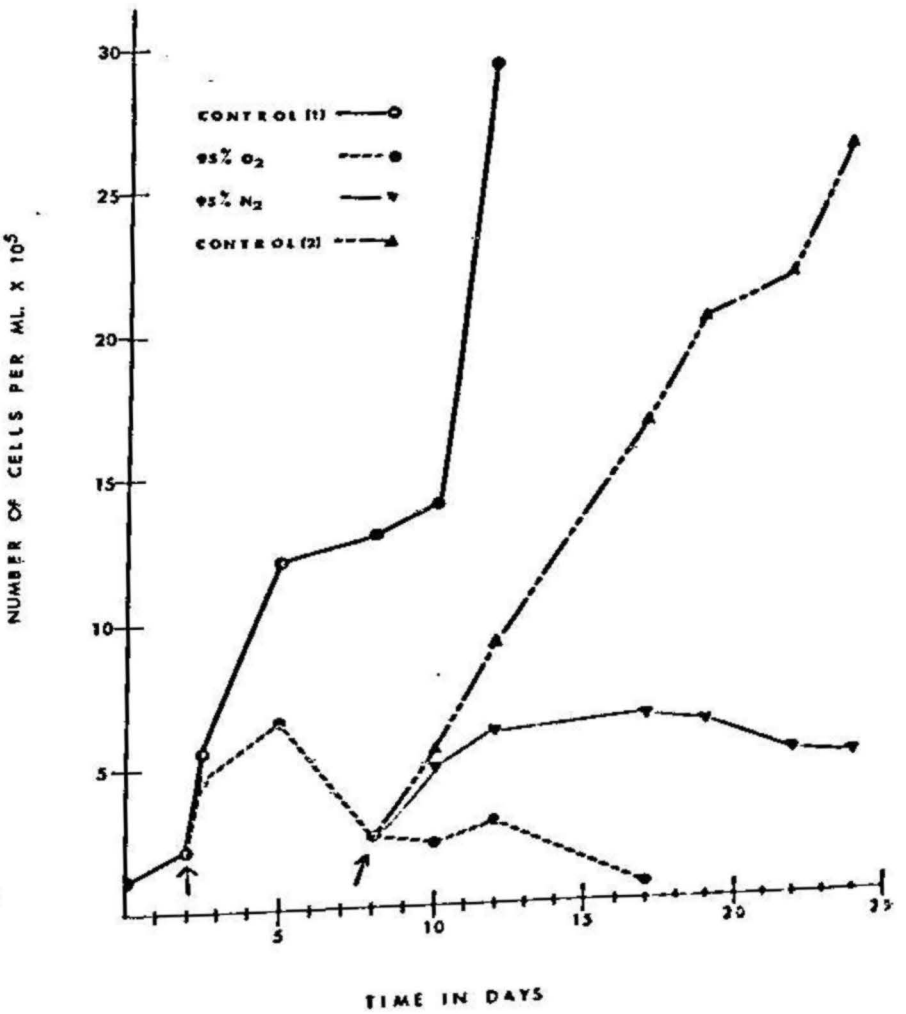


FIGURE 6


CONTROL (1)
95% O_2
95% N_2
CONTROL (2)
NUMBER OF CELLS PER ML. X 10^5
30
25
20
15
10
5
5
10
15
20
25
TIME IN DAYS

4th day after exposure, following which (6-10 days) the cells begin to degenerate progressively, round up, clump together, and detach from the surface of the flask.

In a gaseous environment lacking O_2 (or nearly so), the cells are also apparently damaged but not nearly so much as in the case of high O_2 environments. They reach a low maximum but do not die for some time (over two weeks).

Naturally, the question arises as to the extent of damage in both cases. If the conditions are reversed, will the cells recover? This phase of the project was then our major concern.

In all experiments, control cultures (control 1) were carried along with the experimental. The controls were gasses with 95% air plus 5% CO_2 and in all the experimental cultures 5% CO_2 was also added to the gas mixture.

This project was carried out in a series of investigations. The results will be discussed under the heading of each group of tests conducted.

Growth (Cell Numbers)

High O_2 tension

In the first group of experiments the cells were exposed to 95% O_2 (Fig. 6). On the 6th day following exposure, when the toxic effects were definitely evident, the cells were subjected to different conditions to see whether or not recovery was possible. This was done by replacing the 95%

O_2 of one-third of the experimental cultures with 95% air, one-third with 95% N_2, and allowing the other one-third to continue in the high O_2 concentrations.

At this time cell numbers had decreased markedly and the cells clearly show the effects of high O_2 tension (Fig. 6). However, within two days after the gaseous changes, those cells now exposed to control (control No. 2) treatment begin to return to their usual morphological form although they still contain many vacuoles. The cells now exposed to 95% N_2 also show signs of recovery within the 2 day period following the change; there is an increase in numbers. At the termination of the experiment, those cells now in the "control No. 2" atmosphere are morphologically indistinguishable from the original controls. The cultures subjected to 95% N_2 are quite easily distinguished from the others since the cells become somewhat larger and contain many giant cells.

The cells that had not been changed, but remained exposed to 95% O_2 throughout the remainder of the experiment were dead by the end of 10 days. However, those removed from the 95% O_2 and subjected to control conditions (air plus 5% CO_2) recover appreciably and proliferate at a rate equal to, if not exceeding, that in the original control flasks. Cells taken from the 95% O_2 and exposed to 95% N_2 recover enough so as to increase in number but seem to reach

a plateau (Fig. 6).

$\underline{O_2}$ Lack

A second group of experiments was conducted using the same procedures and techniques as in the first group, except that the experimental cultures were originally gassed with approximately 95% N_2 rather than 95% O_2 as in the first group of experiments (Fig. 7). Growth rate and differences in maximum growth under the different gaseous conditions are quite similar to those obtained in the previous group of experiments.

Cells exposed to an environment of 95% N_2 attain a comparatively low growth maximum. After attaining maximum numbers (11th day), there is a progressive although very slow decrease during the remainder of the experiment. Possibly this may indicate a tendency toward an adaptation to anaerobiosis *in vitro*.

On the 10th day following exposure to a nearly anaerobic environment, the cultures were subjected to the following conditions: one-third of the experimental cultures were exposed to the control environment (95% air plus 5% CO_2); one-third, to 95% O_2; and the remaining one-third, were continued in 95% N_2. The original controls (control No. 1) remained in air-CO_2 until termination of the experiment.

The cells (from 95% N_2) now exposed to control

FIGURE 7. Compilation of 3 experiments showing growth of Low Line cells under "normal" conditions (95% air plus 5% CO_2) and in 95% N_2 plus 5% CO_2 (0% O_2) and the effects of eventual exposure of the "damaged" cells under the latter conditions to air or 95% O_2. Arrow to the left indicates the time the cells were exposed to 95% N_2; the other, the time of the gas changeover as indicated.

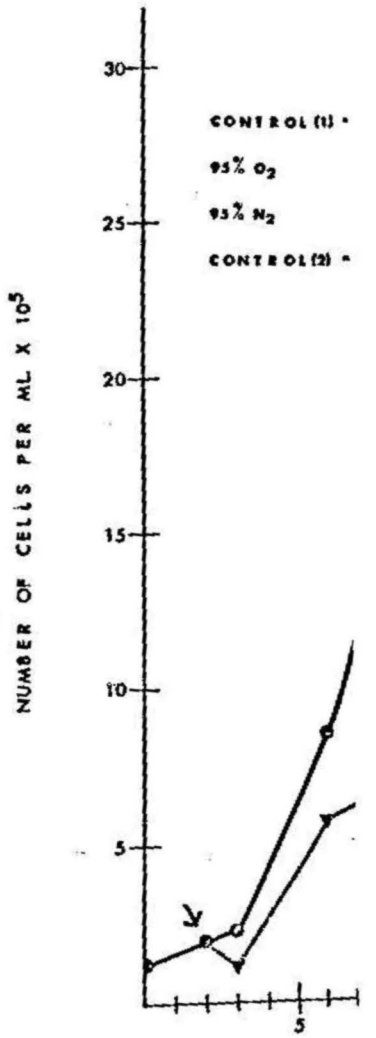
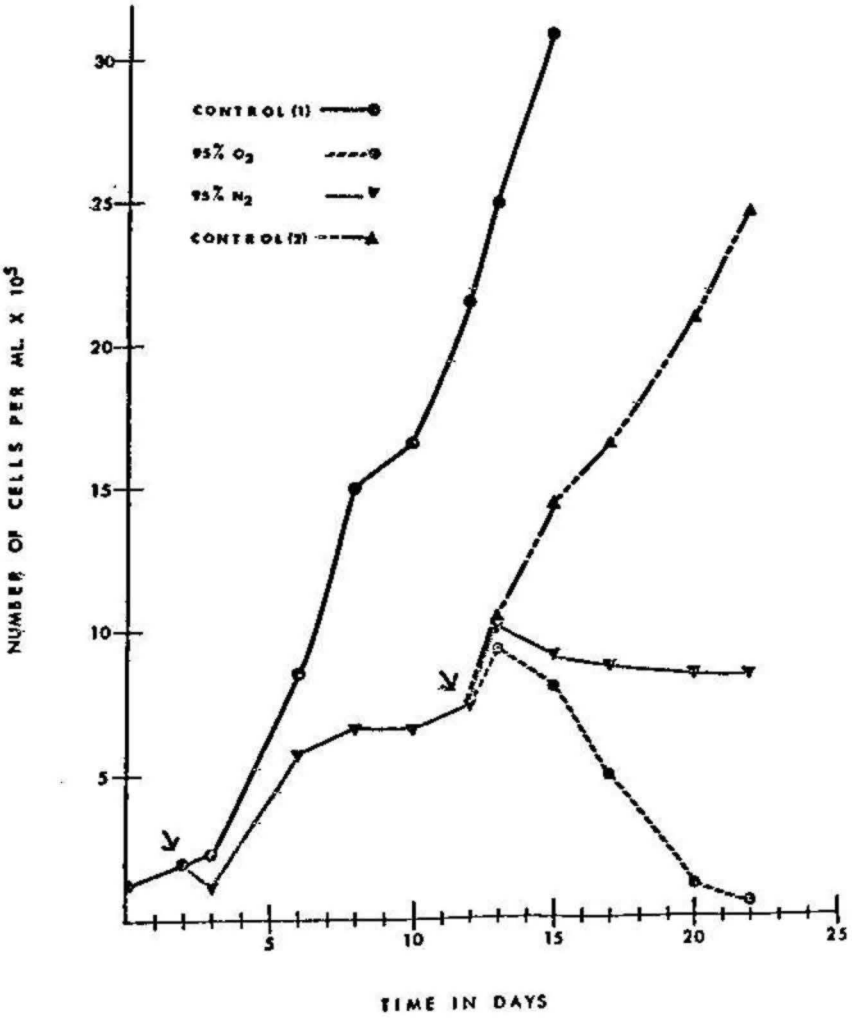


FIGURE 7


CONTROL (1)
95% O_2
95% N_2
CONTROL (2)
NUMBER OF CELLS PER ML X 10^5
30
25
20
15
10
5
5
10
15
20
25
TIME IN DAYS

environment (control No. 2) immediately begin to proliferate at a faster rate, matching the growth rate of the original controls (control No. 1) within the first day. Those now exposed to 95% O_2 soon show the effects of the toxic environment and proliferation decreased markedly (Fig. 7).

Glucose Uptake and Lactic Acid Production

High O_2 tension

The same general pattern seen in cellular proliferation (Fig. 6) was also noted in regards to the metabolism of the cells under high O_2 tensions (Figs. 8 and 9). Glucose uptake and lactic acid (L.A.) production of the cells exposed to 95% O_2 increase steadily for the duration of the experiment. Those cells first subjected to 95% O_2 but now exposed to 95% N_2 and those exposed to 95% air show a marked decrease in rate of glycolysis by the second day following the change-over (Figs. 8 and 9). Cells from 95% O_2 now cultured in the control environment show a decrease in their glucose uptake and L.A. production to such an extent that at the termination of the experiment their glycolytic capacities are indistinguishable from those of the original controls (Figs. 8 and 9). Those cultures now exposed to 95% N_2 decrease their rate of glucose uptake and L.A. production sharply at first, then reach a plateau for the duration of the experiment. At no time does this glycolytic rate approach the low value of the original controls.

O_2 Lack

Glucose uptake and L.A. production in cells exposed to 95% N_2 (Figs. 10 and 11) also follows a similar pattern to

FIGURE 8. Compilation of 3 experiments showing glucose utilization of Low Line cells exposed to control (95% air plus 5% CO_2) and 95% O_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter condition to air or 95% N_2 plus 5% CO_2. Arrow to the left indicates the time the cells were exposed to 95% N_2; the other, the time of the gas changeover as indicated.

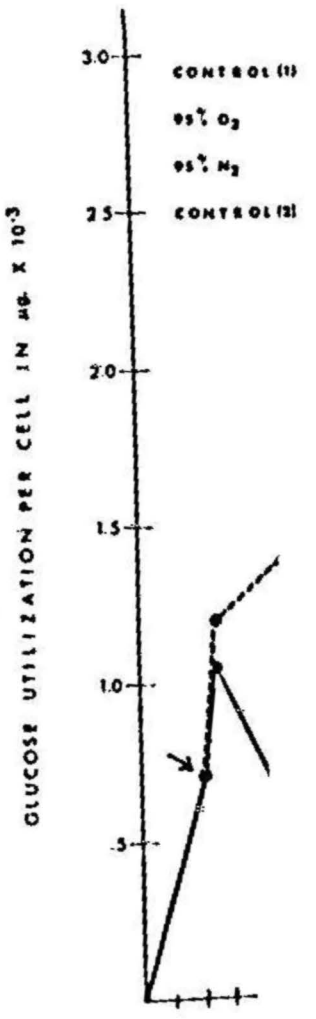
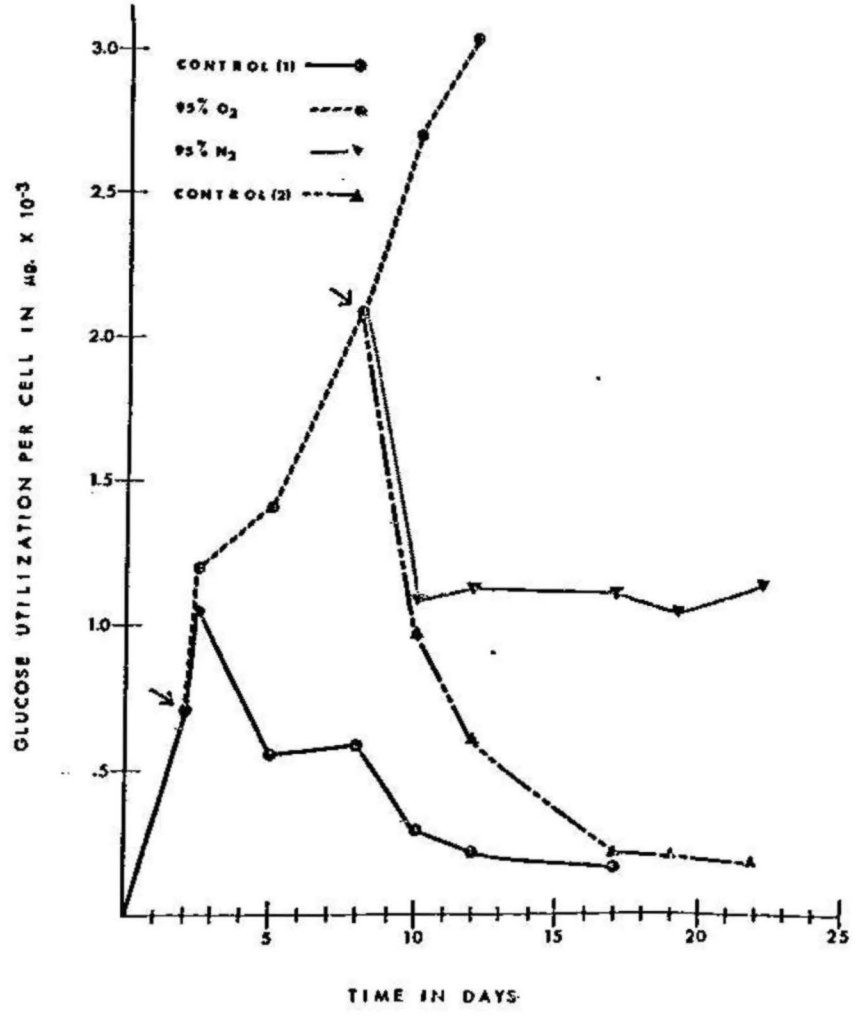


FIGURE 8


CONTROL (1)
95% O_2
95% N_2
CONTROL (2)
GLUCOSE UTILIZATION PER CELL IN µg. X 10^{-3}
3.0
2.5
2.0
1.5
1.0
.5
5
10
15
20
25
TIME IN DAYS

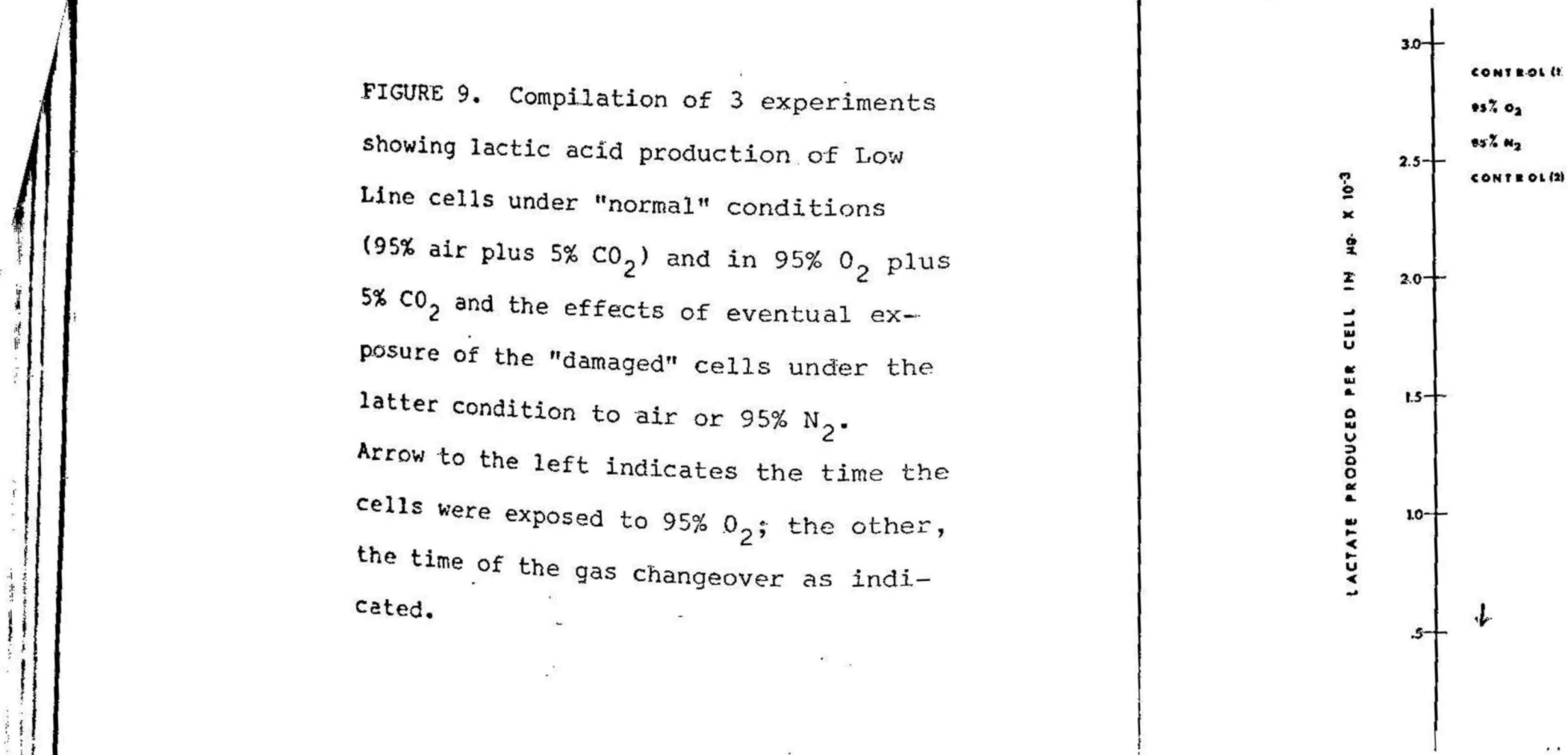


FIGURE 9. Compilation of 3 experiments showing lactic acid production of Low Line cells under "normal" conditions (95% air plus 5% CO_2) and in 95% O_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter condition to air or 95% N_2. Arrow to the left indicates the time the cells were exposed to 95% O_2; the other, the time of the gas changeover as indicated.

FIGURE 9

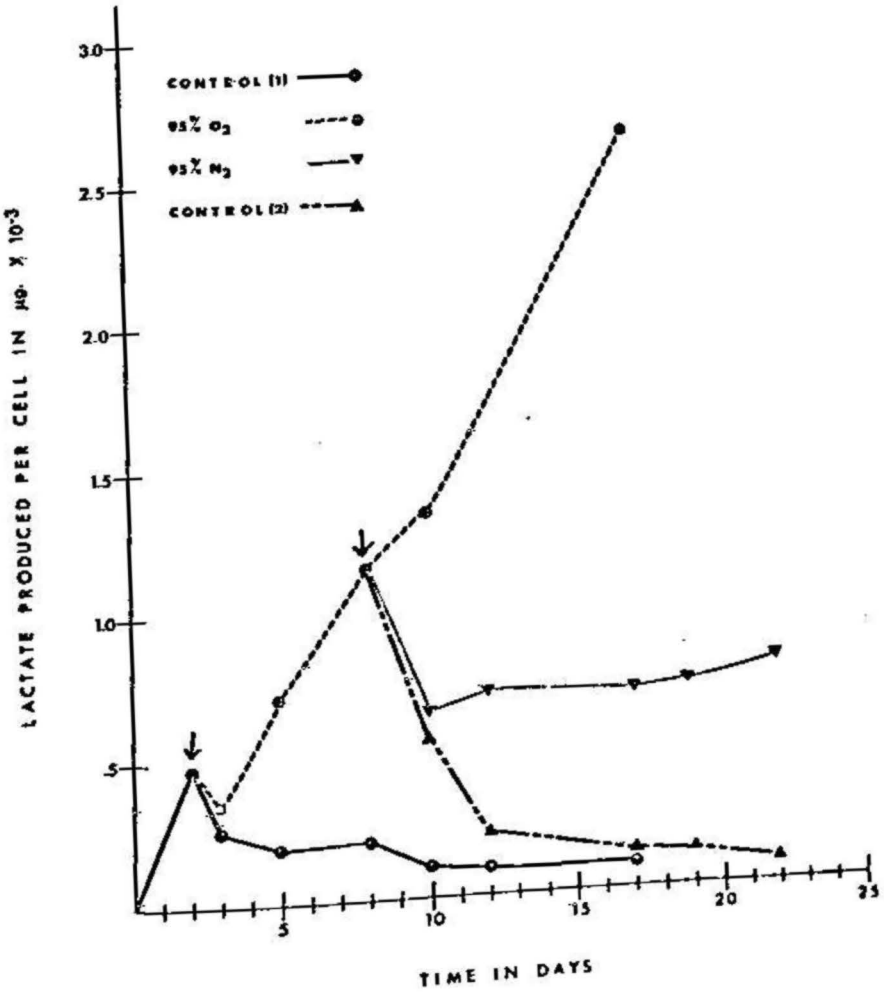

CONTROL (1)
95% O_2
95% N_2
CONTROL (2)
LACTATE PRODUCED PER CELL IN µg. X 10^{-3}
3.0
2.5
2.0
1.5
1.0
.5
5
10
15
20
25
TIME IN DAYS

FIGURE 10. Compilation of 3 experiments showing glucose utlization of Low Line cells under control conditions (95% air plus 5% CO_2) and to 95% N_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter condition to air or 95% O_2 plus 5% CO_2. Arrow to the left indicates the time the cells were exposed to 95% N_2; the other, the time of the gas changeover as indicated.

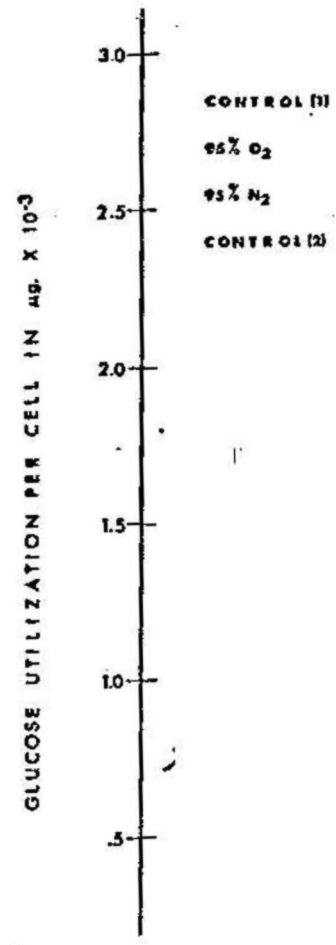


FIGURE 10

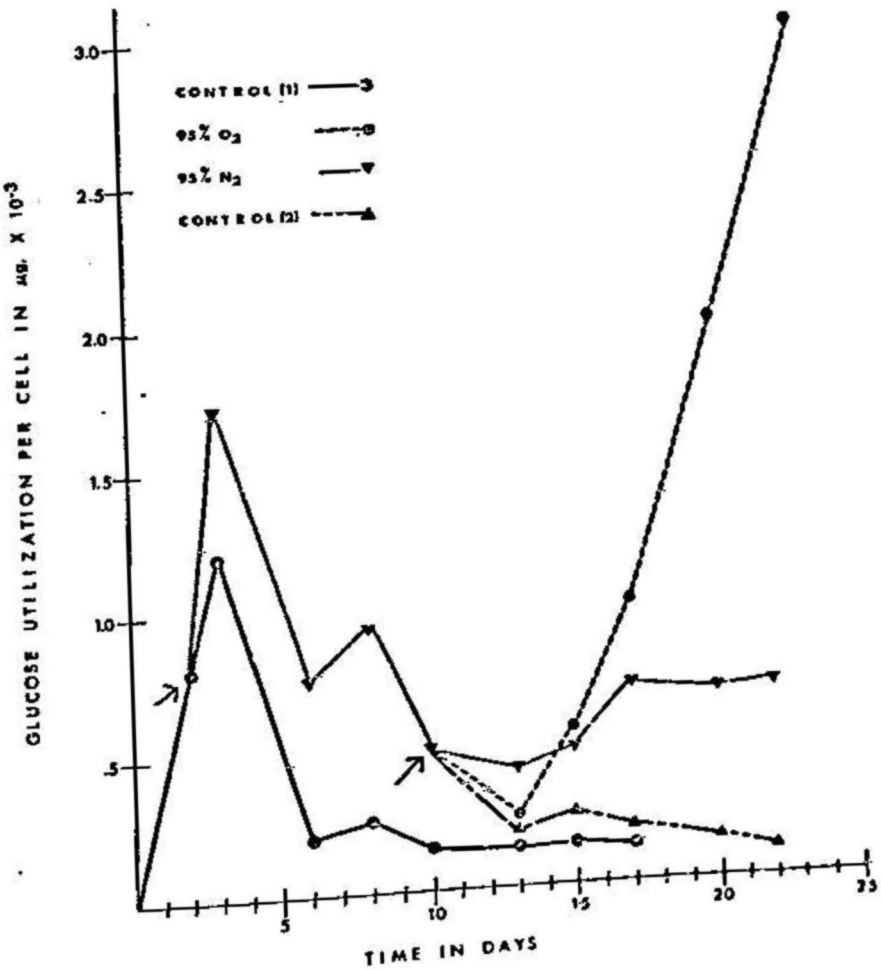

CONTROL (1)
95% O_2
95% N_2
CONTROL (2)
GLUCOSE UTILIZATION PER CELL IN µg. X 10^{-3}
3.0
2.5
2.0
1.5
1.0
.5
5
10
15
20
25
TIME IN DAYS

FIGURE 11. Compilation of 3 experiments showing lactic acid production of Low Line cells under control conditions (95% air plus 5% CO_2) and in 95% N_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter condition to air or 95% O_2. Arrow to the left indicates the time the cells were exposed to 95% N_2; the other the time of the gas changeover as indicated.

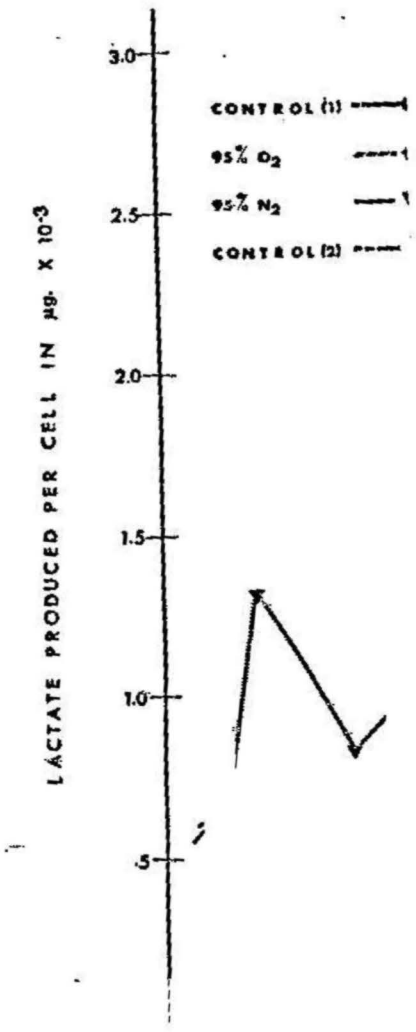
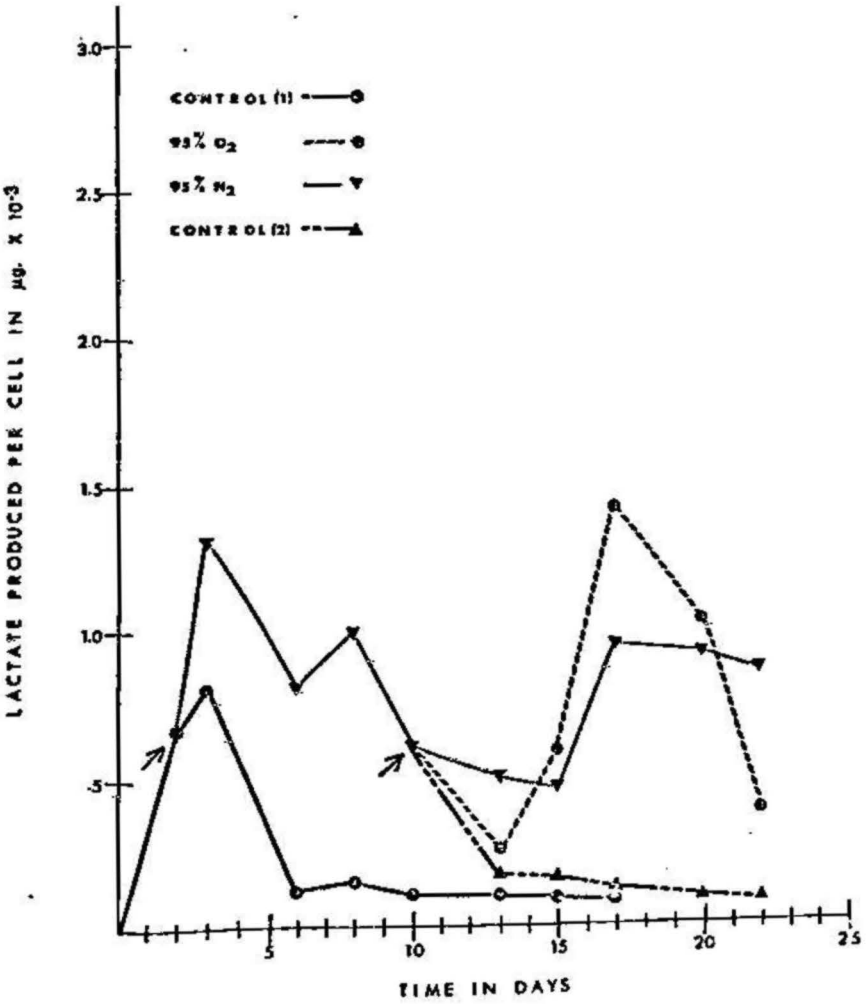


FIGURE 11


CONTROL (1)
95% O_2
95% N_2
CONTROL (2)
LACTATE PRODUCED PER CELL IN µg. X 10^{-3}
3.0
2.5
2.0
1.5
1.0
.5
5
10
15
20
25
TIME IN DAYS

that of their growth curves (Fig. 7). In 95% N_2 the glucose uptake and L.A. production is somewhat higher than in the control cultures, and it remains consistently high during the 20 days of the experiment. Changing the 95% N_2 environment to 95% O_2 causes a great increase in both glucose uptake and L.A. production of the cells, although after 7 days the L.A. production decreases while the glucose uptake increases sharply. Actually, it is doubtful that any significance can be applied to the glucose and L.A. results for the cells in 95% O_2 during the last four days of the experiment. Cells put in the control environment from the 95% N_2 immediately show a decrease in glucose utilization and L.A. production, and within 3 days approach the rate of the original control cultures.

From these initial results it was evident that recovery from the toxic environments to which the cells were subjected did occur and that growth, glucose uptake, and L.A. production in the control-2 cultures returned to a pattern seen in the control-1 cultures a short time after the change-over. From these experiments further investigations were carried out to ascertain whether or not other systems were involved in high or low O_2 environments and their possible recovery when the toxic conditions are removed. Since full recovery apparently was achieved only in the cells from those cultures in which 95% O_2 or 95% N_2 was

replaced by 95% air (control conditions), further studies were henceforth limited to this type of experimentation.

Pyruvic Acid Production

In cells exposed to 95% O_2 the pyruvic acid production per cell increases sharply after one day exposure and remains high throughout the experiment (Fig. 12). Those cells first subjected to 95% O_2 and then exposed to 95% air (control-2) show a marked decrease in rate of pyruvic acid production. At the termination of the experiment it is nearing that of the original controls (control-1). In cultures exposed to 95% N_2 the rate of pyruvic acid production is somewhat higher than the control-1 rate but is no where near that observed in the cultures exposed to 95% O_2. Those cells subjected to 95% N_2 but now exposed to 95% air (control-3) decrease their production of pyruvic acid so that at the termination of the experiment their rate is similar to that of the original controls. The cells growing under a 95% air environment (control-1) exhibit a pyruvic acid production curve similar to the glucose uptake and lactic acid production curves seen earlier. That is, after an initial elevation in pyruvic acid production the amount produced per cell decreases with culture age to about the 6-8th day and then levels off and remains at this level the rest of the experiment.

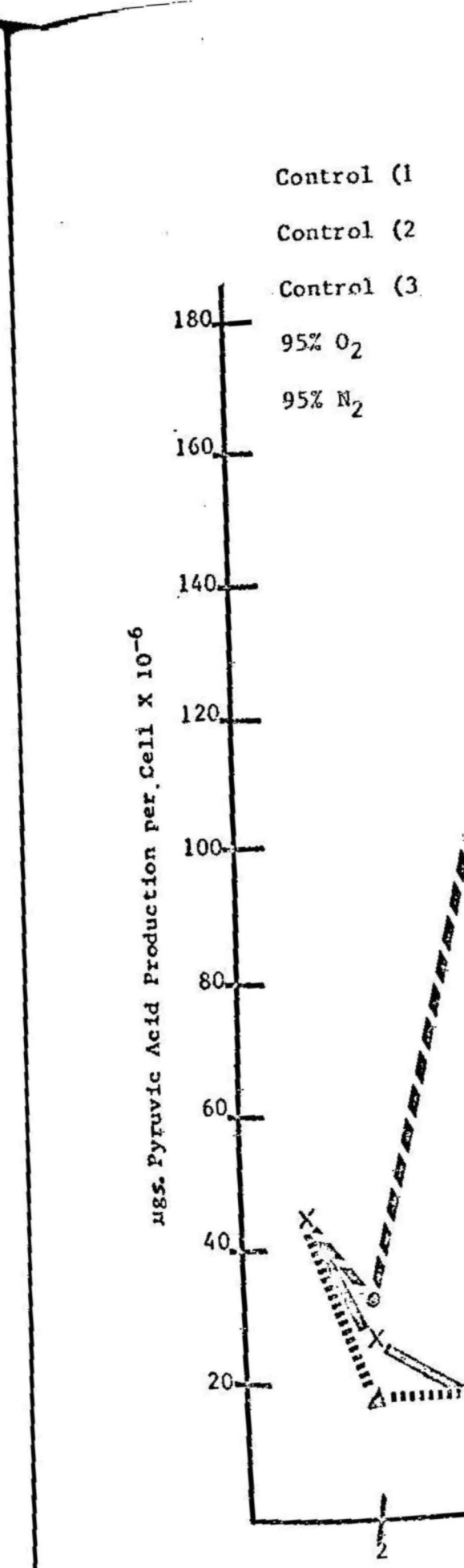
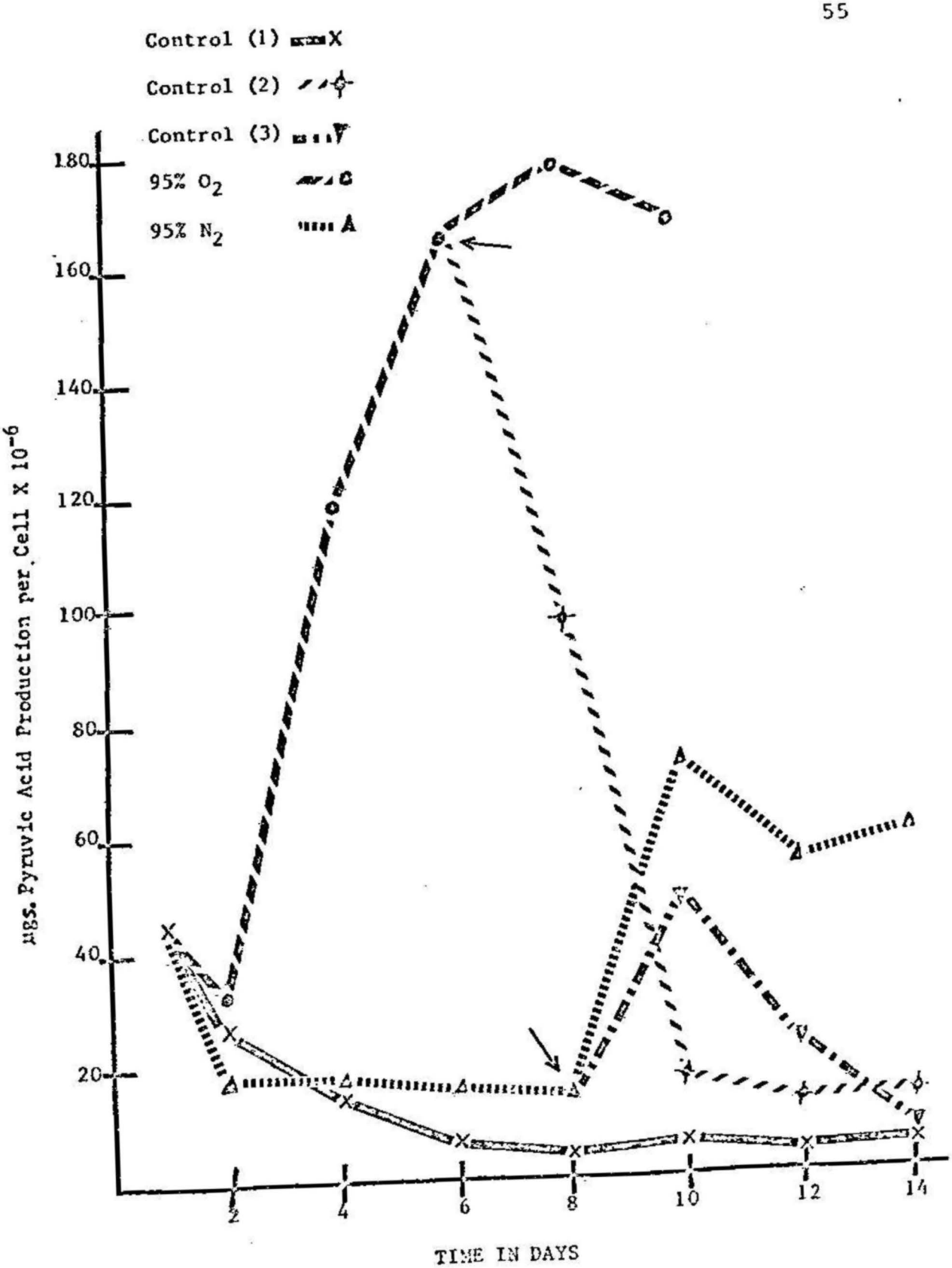


FIGURE 12. Compilation of 3 experiments showing pyruvic acid production of Low Line cells exposed to control conditions (95% air plus 5% CO_2), 95% O_2 plus 5% CO_2 plus 5% CO_2, or 95% N_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter two conditions to an atmosphere of 95% air plus 5% CO_2. Arrows indicate the time of this gas changeover.

FIGURE 12


Control (1) X
Control (2)
Control (3)
95% O_2 C
95% N_2 A
180
160
140
120
100
80
60
40
20
µgs. Pyruvic Acid Production per Cell X 10^{-6}
2
4
6
8
10
12
14
TIME IN DAYS

Acid and Alkaline Phosphatase Activity

Alkaline phosphatase activity was ascertained by histochemical and clorimetric analyses on the medium in which the cells grow, on the cells themselves, and on cell homogenates. At no time was there any evidence of alkaline phosphatase activity within the limits of the tests employed. This fact will be considered in the discussion.

Acid phosphatase activity per mμg protein (Fig. 13) decreases gradually in those cells cultivated in 95% air (control-1) from the high found at the beginning of an experiment until the 6-8th day of culture when it levels off and then remains rather constant during the rest of the experiment. In those cells subjected to 95% O_2 a steady increase in the acid phosphatase activity occurs after the 1st day of exposure. Cultures previously exposed to 95% O_2 but now subjected to 95% air (control-2) exhibit a decrease in the amount of acid phosphatase and at the termination of the experiment the rate approaches that of the original controls although still somewhat higher. Cells cultivated in 95% N_2 show an acid phosphatase activity somewhat intermediate between the original control and those exposed to 95% O_2. Those subjected to 95% N_2 and now exposed to 95% air (control-3) show a marked decrease in the amount of acid phosphatase which approaches that found in the original control cultures.

FIGURE 13. Compilation of 4 experiments showing acid phosphatase activity of Low Line cells exposed to control conditions (95% air plus 5% CO_2), 95% O_2 plus 5% CO_2, or 95% N_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter two conditions to an atmosphere of 95% air plus 5% CO_2. Arrows indicate the time of this change-over. Acid phosphatase activity is expressed in Sigma units. One Sigma unit of phosphatase will liberate 1 μM of p-Nitrophenol per hour.

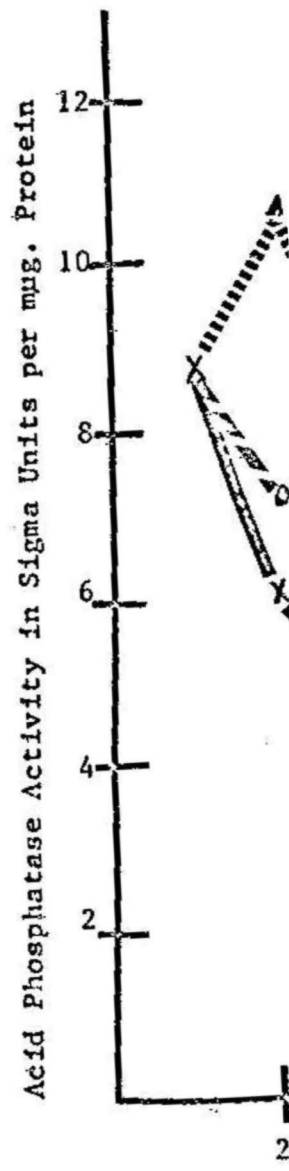
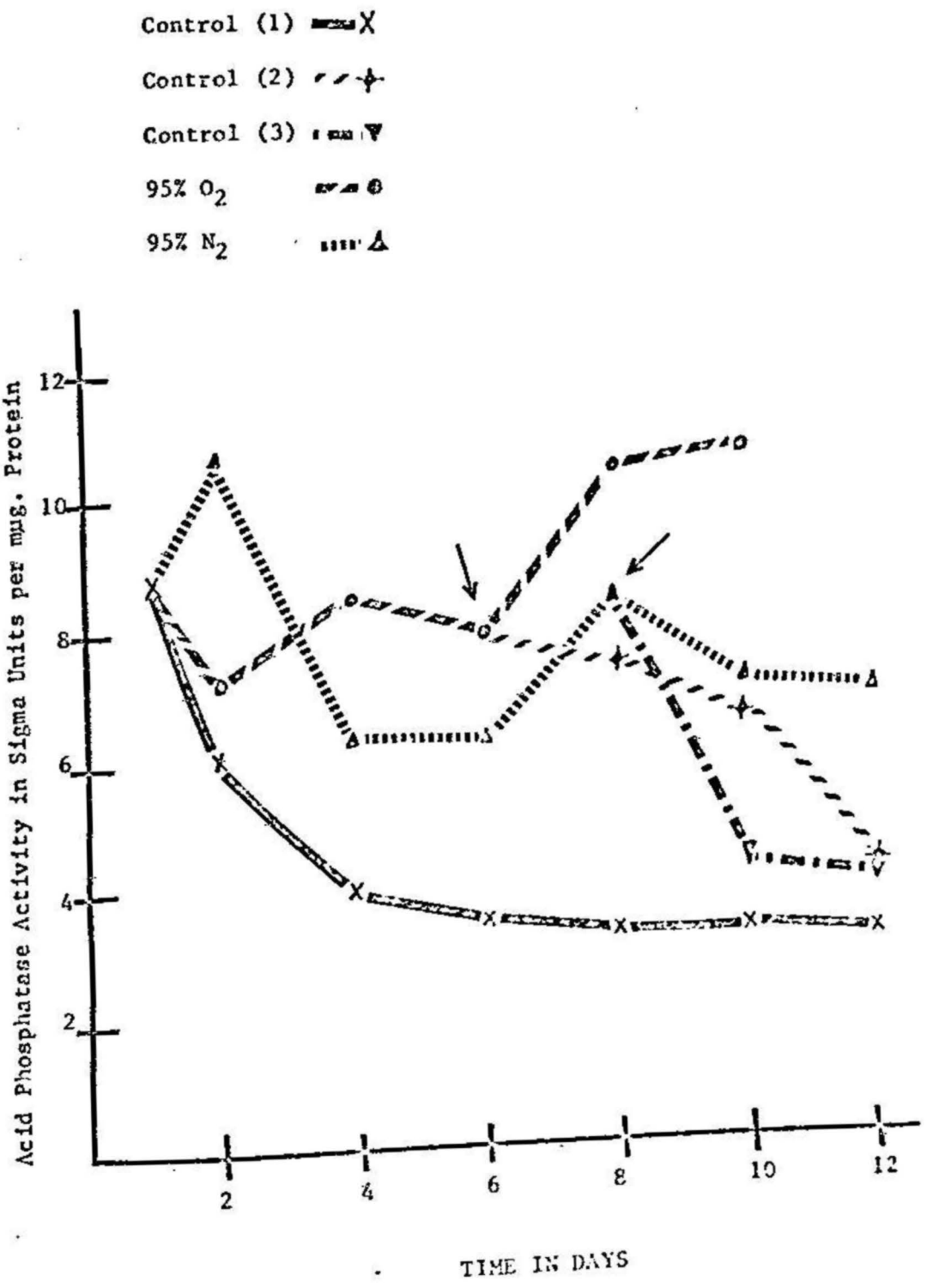


FIGURE 13


Control (1)
Control (2)
Control (3)
95% O_2
95% N_2
Acid Phosphatase Activity in Sigma Units per mg. Protein
12
10
8
6
4
2
2
4
6
8
10
12
TIME IN DAYS

Lactic Dehydrogenase (LDH) Activity)

LDH activity which is greatest immediately following replication (day 1), is expressed in Berger-Broida (B-B) units per ug protein (Fig. 14). One B-B unit will reduce 4.8×10^{-4} µmoles of pyruvic acid per minute at 25°C. (Berger and Broida, 1967). From this high point the activity diminishes in both control and experimental cultures. In cells exposed to 95% air (control-1) the decrease in LDH activity is most rapid and continues to taper off until about the 6-8th day of culture at which time the amount of activity levels of to around 10 B-B units for the duration of the experiment. In cells subjected to 95% O_2, LDH activity also diminishes from the initial high at day 1, but not nearly to the extent as that found in the original controls. At no time does the LDH activity of the cells in 95% O_2 go below 25 B-B units. In cultures first exposed to 95% O_2 then placed in 95% air (control-2), LDH activity decreases to such an extent that at the termination of the experiment it is similar to that of the original controls (about 10 B-B units). Cells in 95% N_2 also exhibit a reduction in LDH activity from day 1 but reach a plateau in their activity (approximately 20 B-B units) about the 6 day of exposure. This level is maintained for the duration of the experiment. If the 95% N_2 is replaced with 95% air (control-3) LDH activity is decreased so that in 4 days it compares to that of the original control cultures.

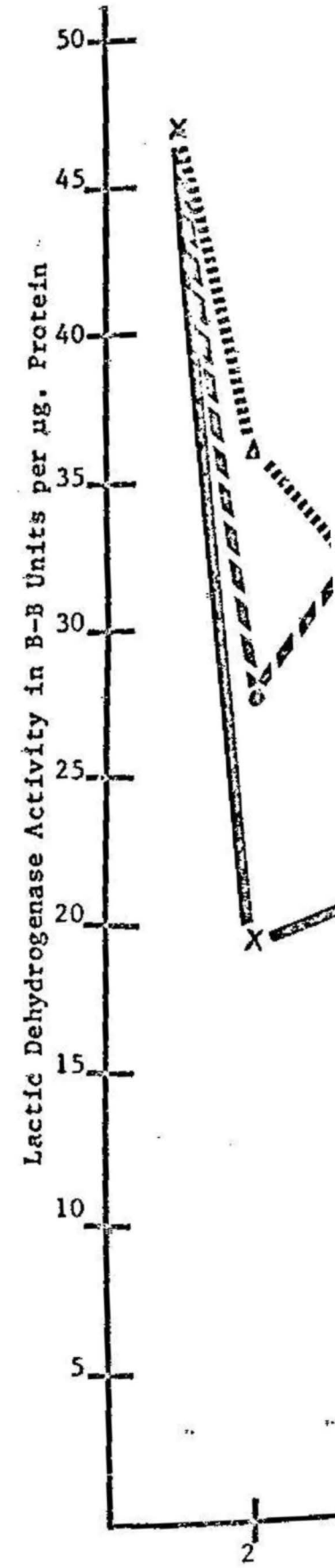
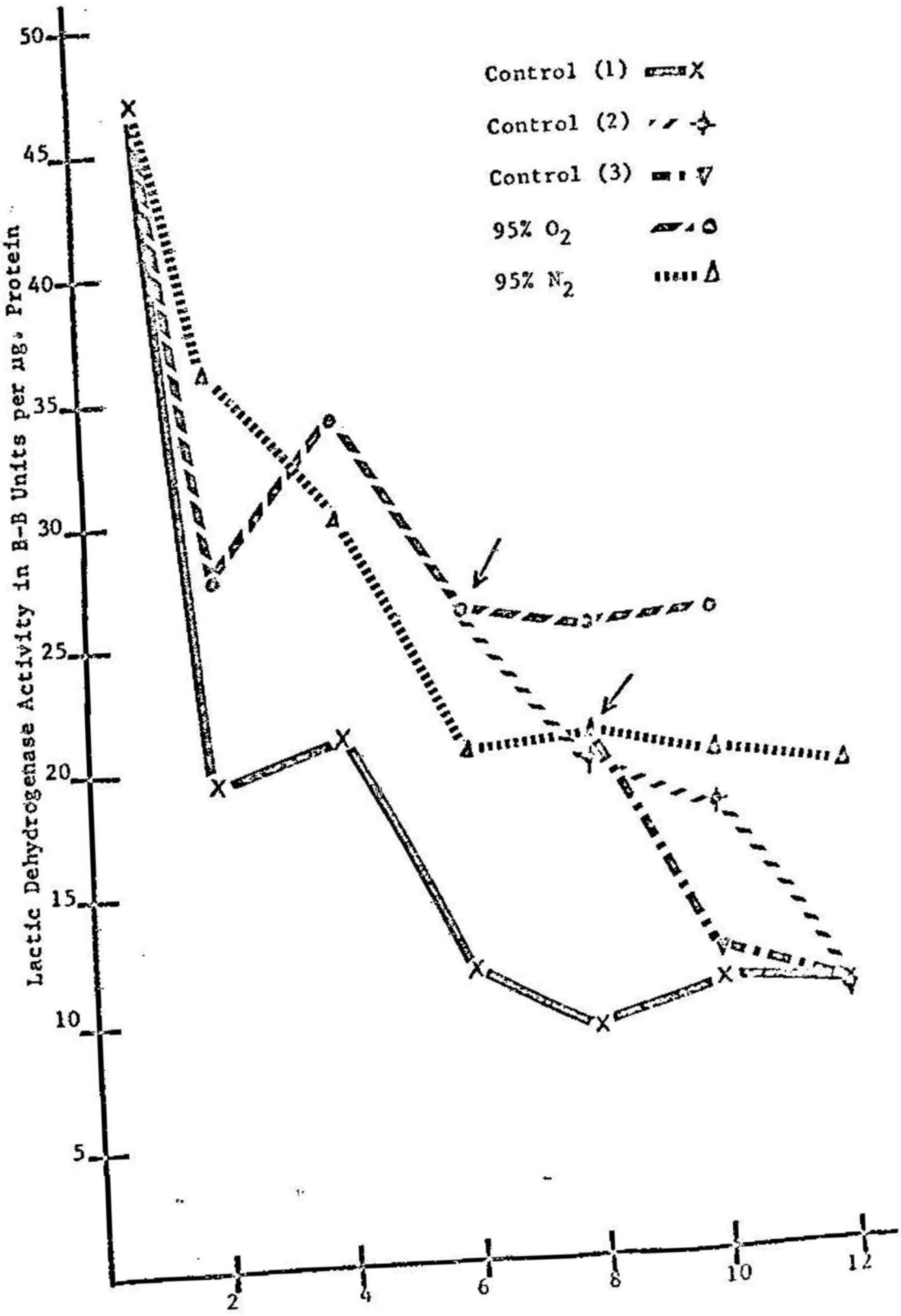


FIGURE 14. Compilation of 4 experiments showing lactic dehydrogenase activity of Low Line cells exposed to control conditions (95% air plus 5% CO_2), 95% O_2 plus 5% CO_2; or 95% N_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter two conditions to an atmosphere of 95% air plus 5% CO_2. Arrows indicate the time of this gas changeover.

FIGURE 14


Control (1)
Control (2)
Control (3)
95% O_2
95% N_2
Lactic Dehydrogenase Activity in B-B Units per µg. Protein
50
45
40
35
30
25
20
15
10
5
2
4
6
8
10
12
TIME IN DAYS

Deoxyribonucleic Acid (DNA)

DNA was ascertained and then calculated in two different ways. First, the total amount of DNA in the cells per ml. of culture medium in which the cells had grown, and second the total amount of DNA per cell.

The pattern of the graph in which is plotted DNA content per ml. (Fig. 15-A), as expected, follows that seen in the various growth curves previously presented. In cultures exposed to 95% air (control-1) the amount of DNA per ml. increases with culture age. The cells in 95% O_2 show a slight increase in DNA per ml. during the first 5-6 days of exposure but then possibly due to the cytotoxic effect of the environment to which they have been subjected it diminishes drastically and becomes exceedingly low. In cells gassed with 95% O_2 and then placed in 95% air (control-2), the DNA per ml. increases steadily during the remainder of the experiment. Cells in a 95% N_2 environment show a gradual increase in the quantity of DNA per ml. until the 5-6th day of exposure after which it decreases somewhat and then levels off to around 5-6 µg DNA. Those previously treated with 95% N_2 and later (8th day) replaced with 95% air (control-3) demonstrate a sharp rise in DNA per ml. of medium. This rises continues throughout the experiment.

When DNA content is calculated on a per cell basis the results give a somewhat different picture of the events that are occurring (Fig. 15B). In 95% air (control-1) the

FIGURES

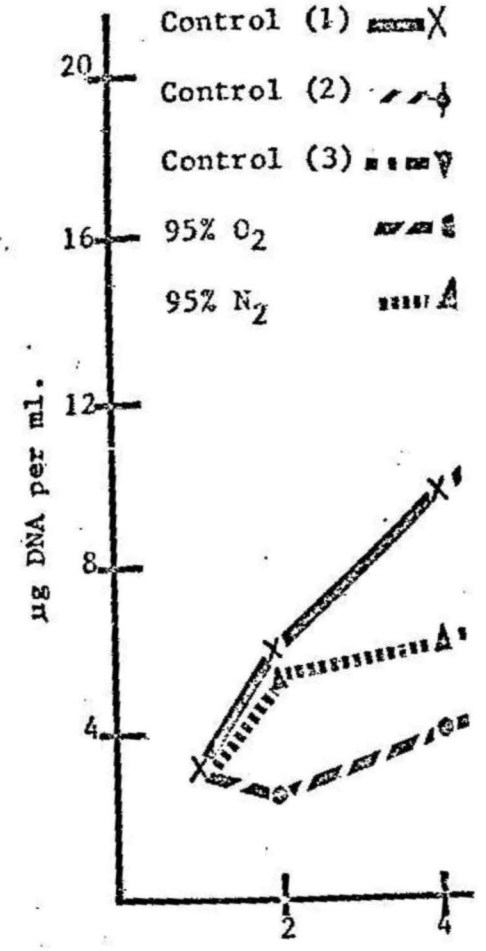


FIGURE 15A. Compilation of 3 experiments showing amount of DNA in Low Line cells exposed to control conditions (95% air plus 5% CO_2), 95% O_2 plus 5% CO_2, or 95% N_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter two conditions to an atmosphere of 95% air plus 5% CO_2. Arrows indicate the time of this change-over. Results are expressed as the amount of DNA found in the cells per ml. of medium in which the cells had grown.

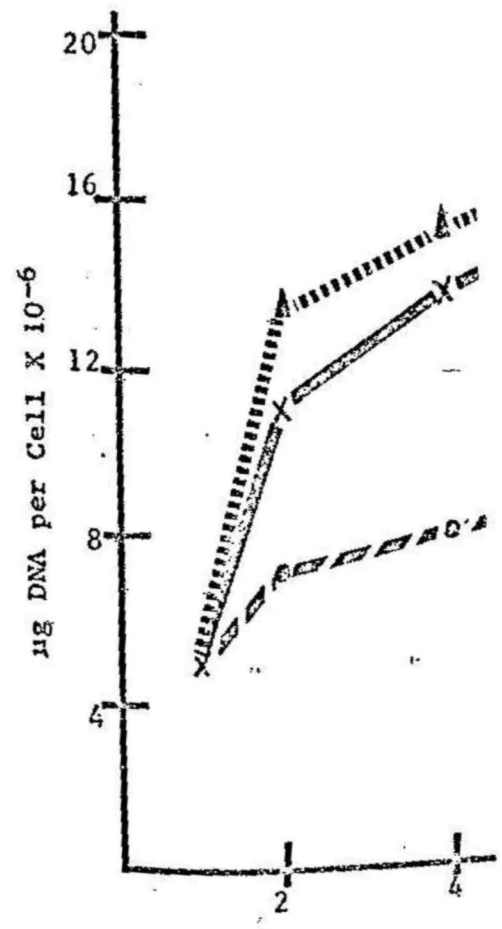


FIGURE 15B. Compilation of 3 experiments showing amount of DNA per cell in Low Line cells exposed to conditions as given in above legend (Fig. 15A).

FIGURES 15A AND 15B

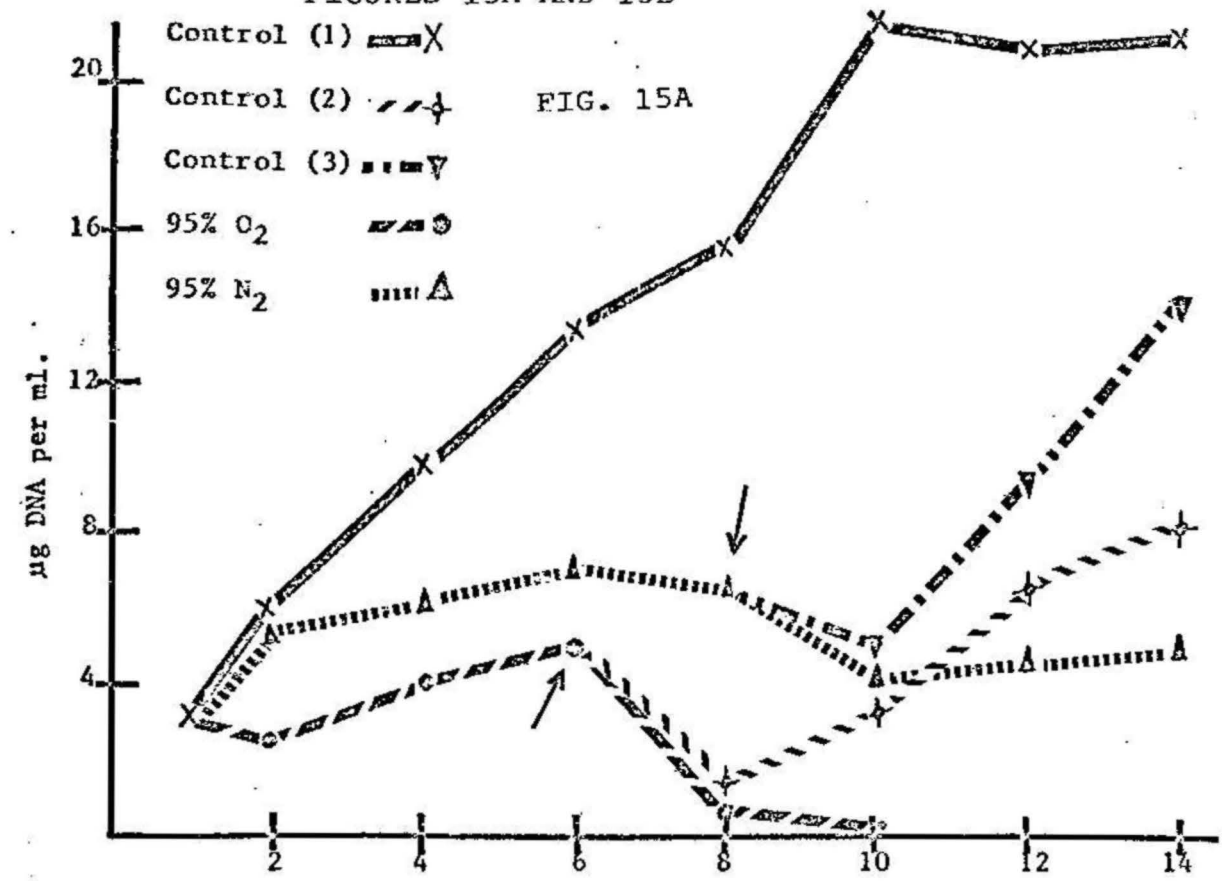


FIG. 15A

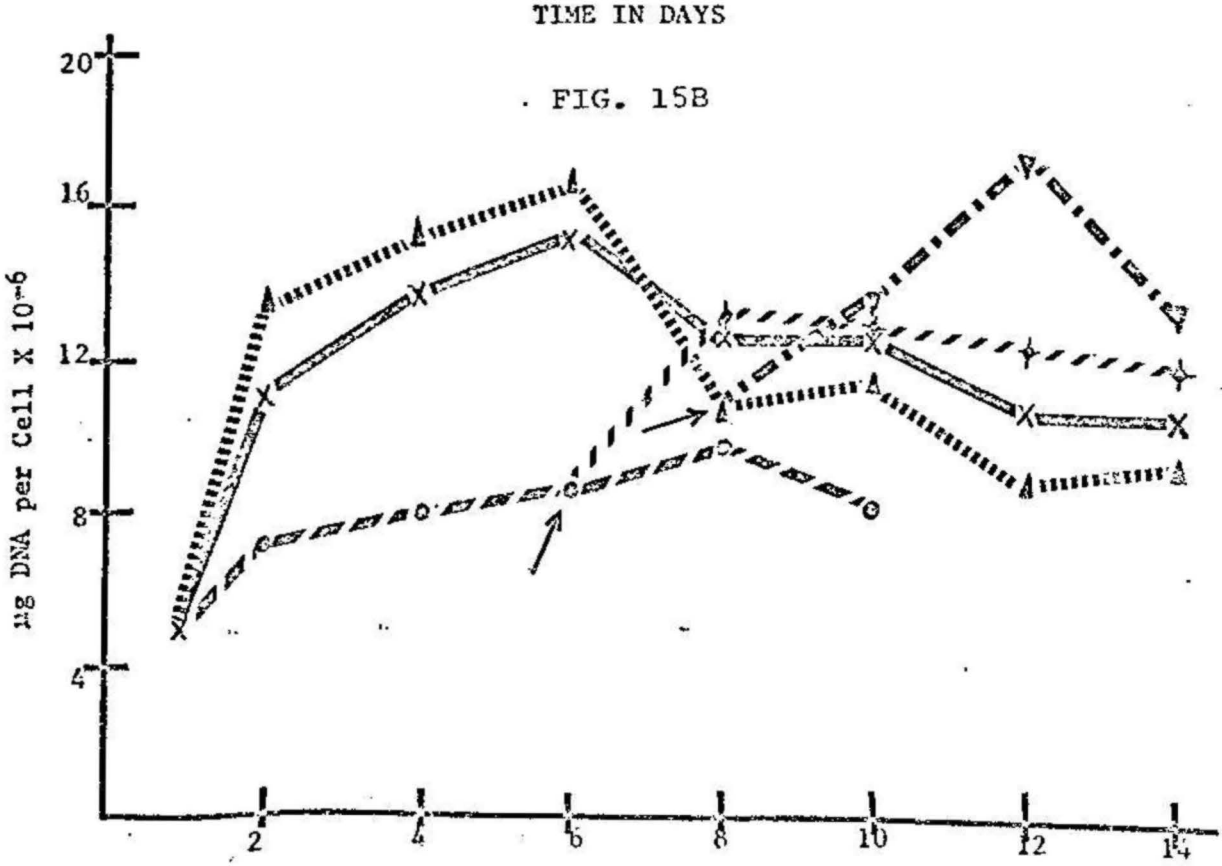


FIG. 15B

DNA per cell increases the first 6 days and then a slight but gradual decrease is observed throughout the rest of the experiment. In cells exposed to 95% O_2 the DNA per cell increases as compared to the controls. In those cells that were exposed to 95% O_2 and later to 95% air (control-2) the DNA per cell increases above that found in the control-1 cultures. The DNA curves for the cells subjected to 95% N_2 follows the same general pattern of that for those in 95% air (control-1) with some important differences. The amount of DNA per cell for the first 6 days is slightly higher in those treated with 95% N_2 but after this time the effect of the continuous anoxic environment results in a severe decrease in this amount and thereafter the level falls somewhat below that found for the original controls. The DNA per cell in cultures in which 95% air replaces 95% N_2 (control-3) increases considerably the first few days after the changeover but by the 14th day it has decreased to a level just slightly above that of the original control cultures (control-1).

Ribonucleic Acid (RNA)

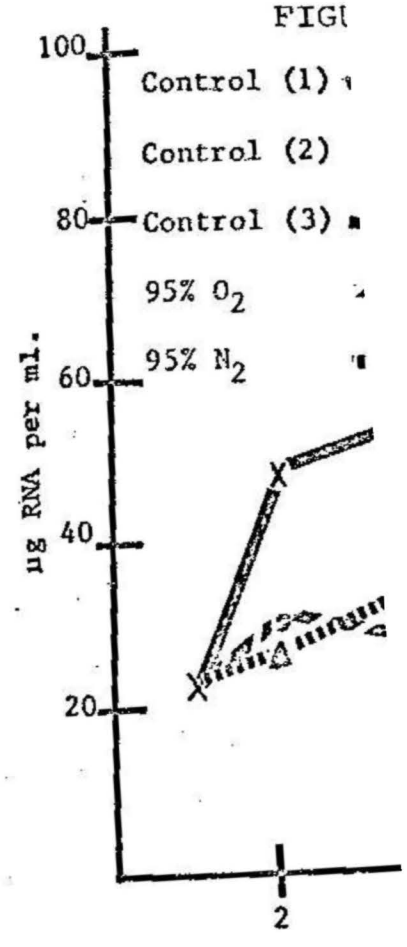
As with DNA the RNA content was ascertained in two different ways: first, the total amount of DNA in the cells per ml. of culture medium in which the cells had grown, and second, the amount of RNA per cell.

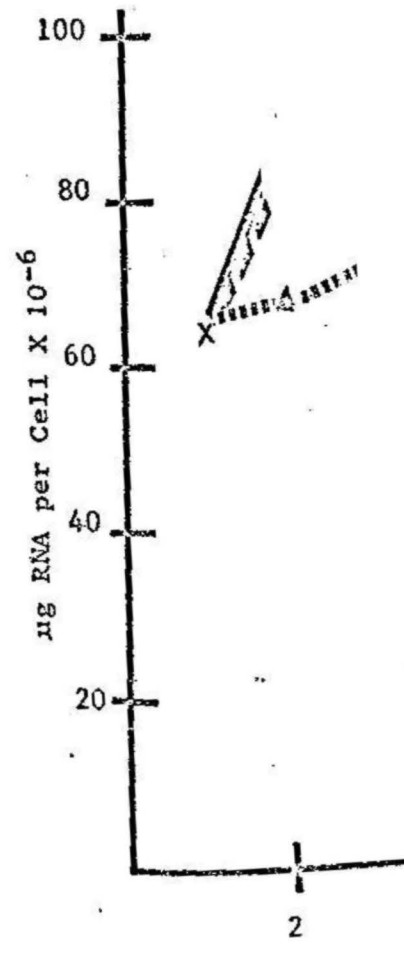
The results of the calculated RNA content per ml. (Fig. 16A) follow the same pattern as those for DNA per ml. (Fig. 15A). The RNA per ml. in cells which were exposed to 95% air (control-1) shows a steady rise throughout the experiment, although it does tend to level off during the last 4 days. In those cultures subjected to 95% O_2 the amount of RNA per ml. of medium remains about the same up until the 6th day when there is a sharp decrease. Cultures exposed to 95% O_2 for 6 days previous to replacing with 95% air (control-2) show a dramatic rise in RNA content per ml. after the first 2 days of the changeover. In those exposed to 95% N_2 the RNA per ml. rises slightly the first 6 days and then decreases somewhat but remains relatively constant thereafter. In cultures previously exposed to 95% N_2 but later replaced with 95% air (control-3) the RNA per ml. takes a sharp rise and at the termination of the experiment it approaches the value found in the original control-1.

When the RNA content is ascertained on a per cell basis it gives a somewhat different picture on the effects

FIGURE 16A. Compilation of 3 experiments showing amount of RNA in Low Line cells exposed to control conditions (95% air plus 5% CO_2), 95% O_2 plus 5% CO_2, or 95% N_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter two conditions to an atmosphere of 95% air plus 5% CO_2. Arrows indicate the time of this changeover. Results are expressed as the amount of RNA found in the cells per ml. of medium in which the cells had grown.

FIGURE 16B. Compilation of 3 experiments showing amount of RNA per cell in Low Line cells exposed to conditions as given in above legend (Fig. 16A).





FIGURES 16A AND 16B

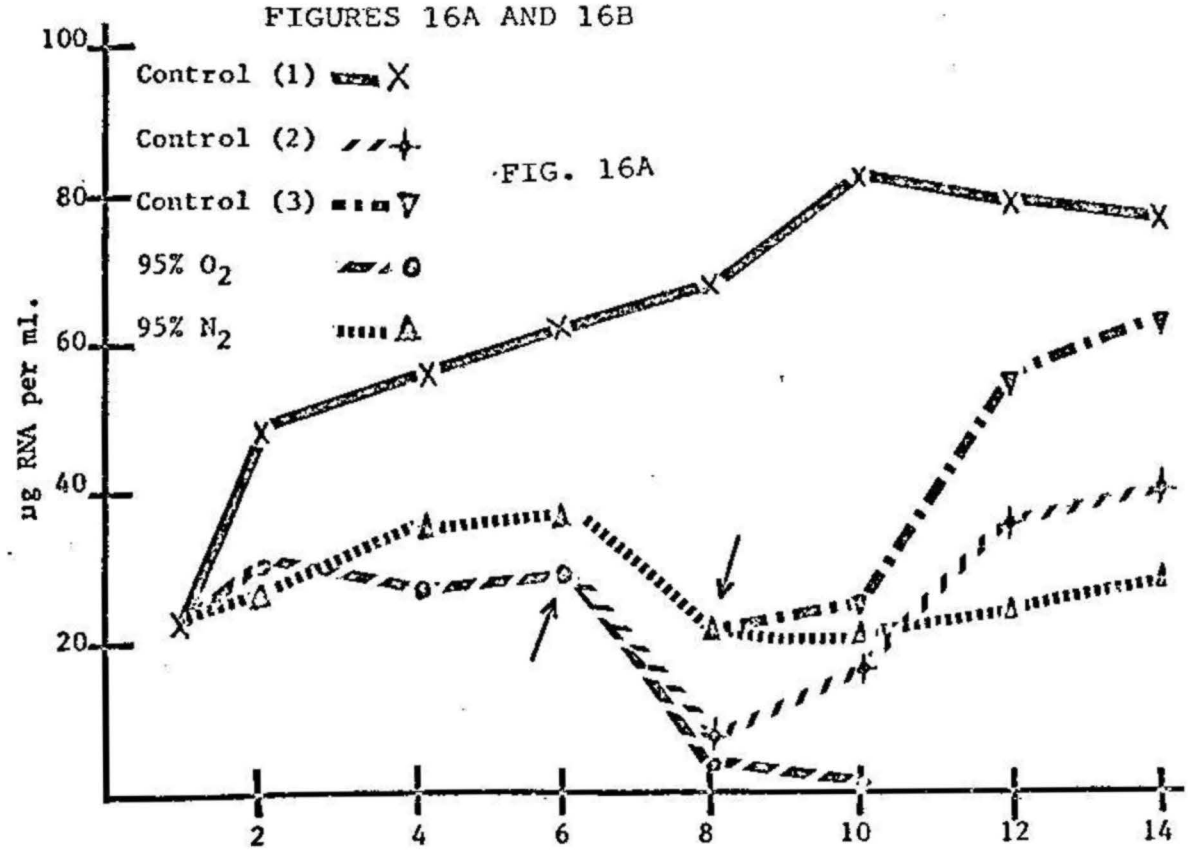


FIG. 16A

TIME IN DAYS

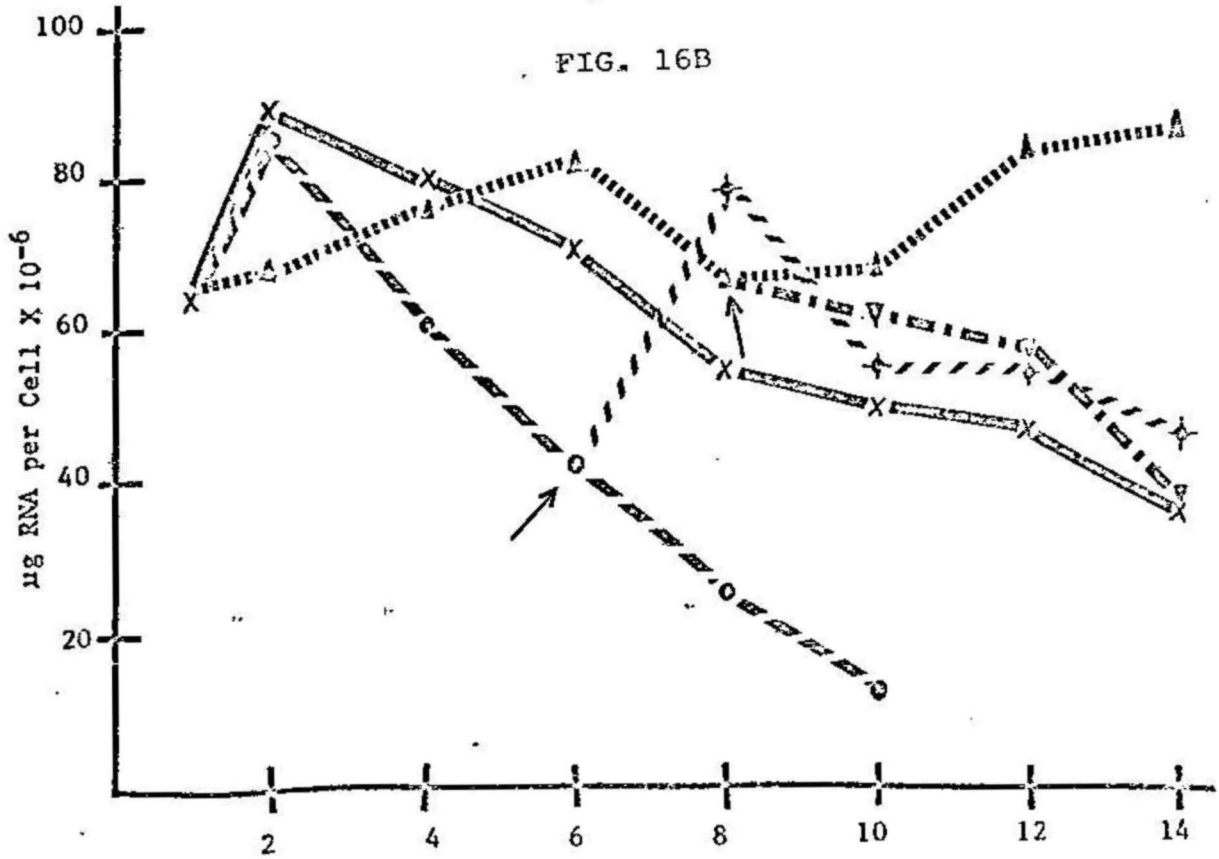


FIG. 16B

of the conditions to which the cells were exposed (Fig. 16B). Control-1 cultures, after an initial rise in the amount of RNA per cell, exhibit a steady decline continuously until the termination of the experiment (14th day). Likewise, cells exposed to 95% O_2 show a decline in the amount of RNA per cell after an initial increase. It should be pointed out that the decline in this case is much more marked than that of control-1. The cultures exposed to 95% O_2 followed by replacement with 95% air on the 6th day (control-2) show a significant rise in RNA per cell the first 2 days after the changeover and then it begins to decline to a rate approaching that of the original controls. Cells in a 95% N_2 environment show a rise in RNA content per cell the first 6 days followed by a decline the next 4 days with a rise again in the latter days of the experiment. At the termination of the experiment these cells contained almost twice the amount of RNA as did the original controls. Cultures first exposed to 95% N_2 followed by 95% air (control-3) on the 8th day show a steady decline in RNA content per cell. At the termination of the experiment the RNA concentration was approximately equal to that of the original control cultures.

Protein

Determination for protein content were made in a similar manner as that for DNA and RNA. That is, (1) the amount of protein in the cells per ml. of culture medium in which the cells had grown, and (2) the amount of protein per cell.

In Figure 17, graphs are presented showing the protein content of the cells per ml. of the culture medium in which cells had grown during a 14 day period. The contour of the curves follows closely that seen in the growth curves (Figs. 6 and 7). The cultures in 95% air (control-1) show a steady increase in total protein per ml. throughout the experiment. In cells exposed to 95% O_2 the total amount of protein increases slightly until day 6 when it decreases markedly until the experiment was terminated. In those previously exposed to 95% O_2 and then returned to 95% air (control-2) the protein per ml. increases rather rapidly for the duration of the experiment. Cells subjected to 95% N_2 show a slight increase in protein per ml. which seems to reach a plateau around the 4-6th day of exposure and remains at this level throughout the experiment. However, if these cultures are now returned to 95% air (control-3) the protein per ml. increases and continues to increase up to the conclusion of the experiment.

The amount of protein per cell was also calculated and the results are presented in Figure 18. The cells of the

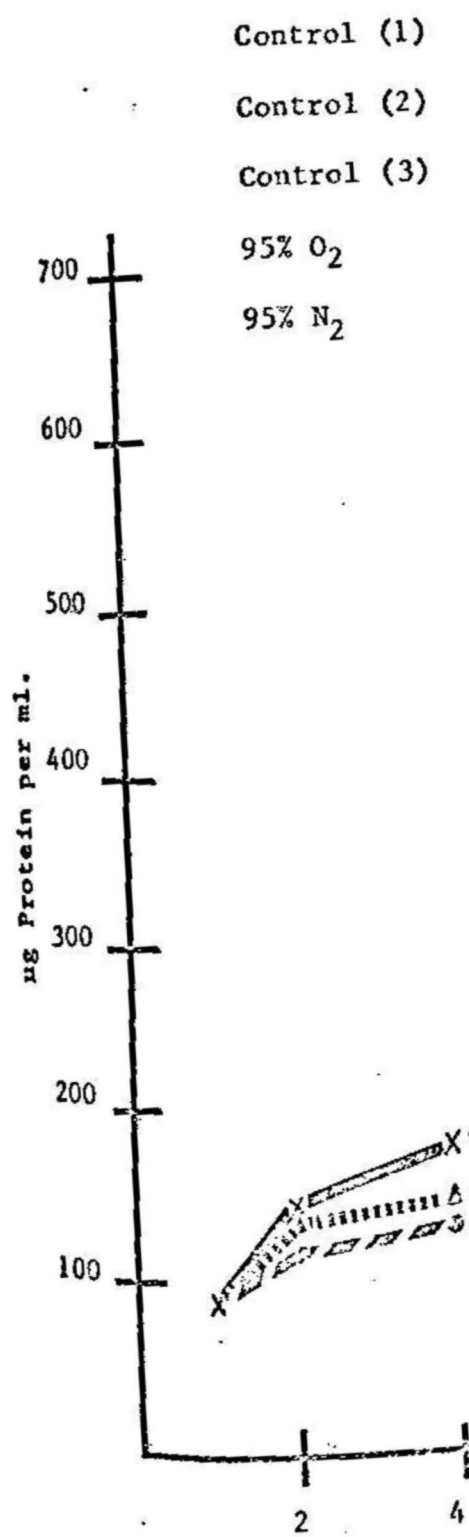
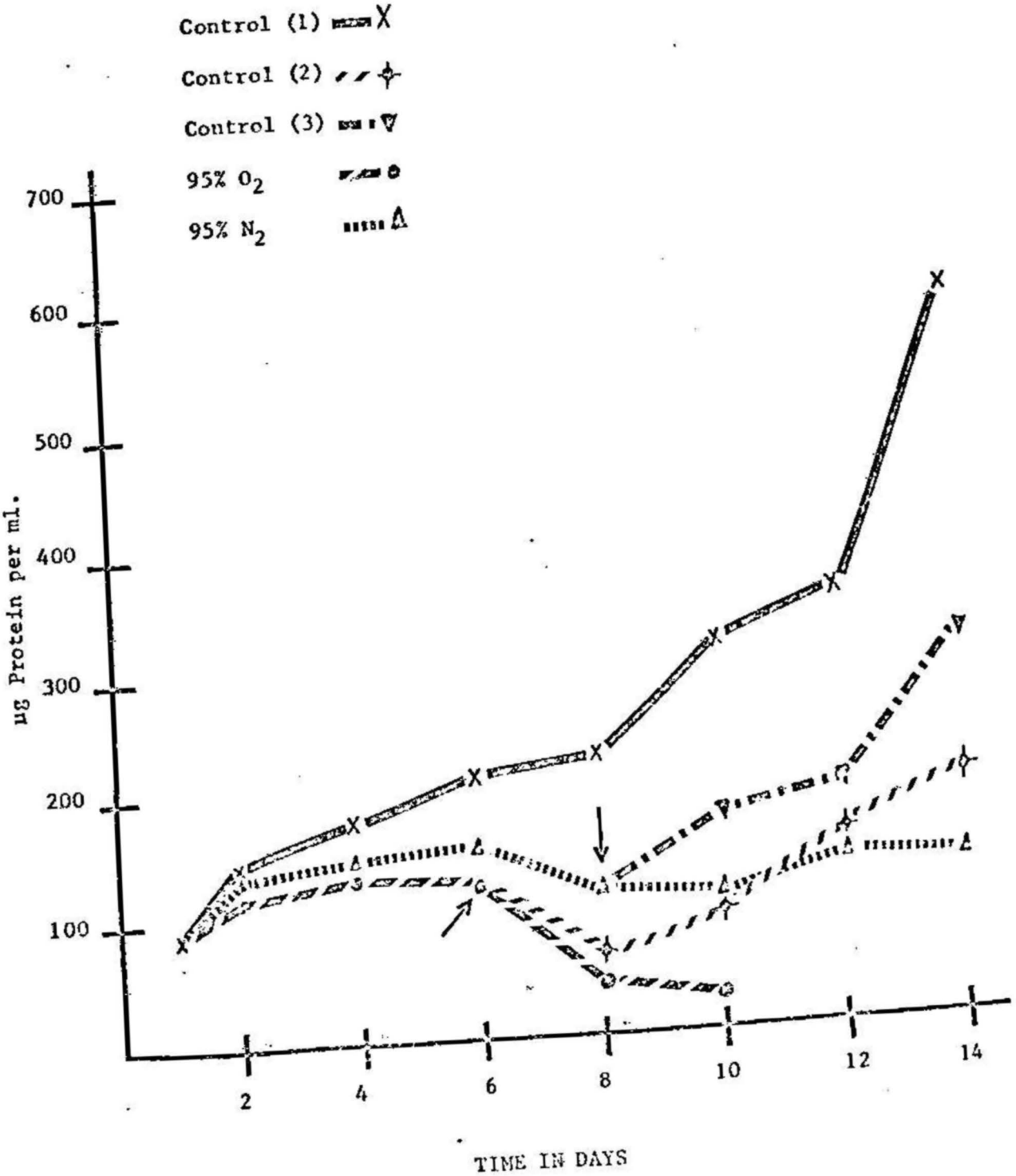


FIGURE 17. Compilation of 3 experiments showing amount of protein in Low Line cells exposed to control conditions (95% air plus 5% CO_2), 95% O_2 plus 5% CO_2, or 95% N_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter two conditions to control conditions. Arrows indicate the time of this changeover. Results are expressed per ml. of medium in which the cells had grown.

FIGURE 17


Control (1)
Control (2)
Control (3)
95% O_2
95% N_2
700
600
500
400
300
200
100
ug Protein per ml.
2
4
6
8
10
12
14
TIME IN DAYS

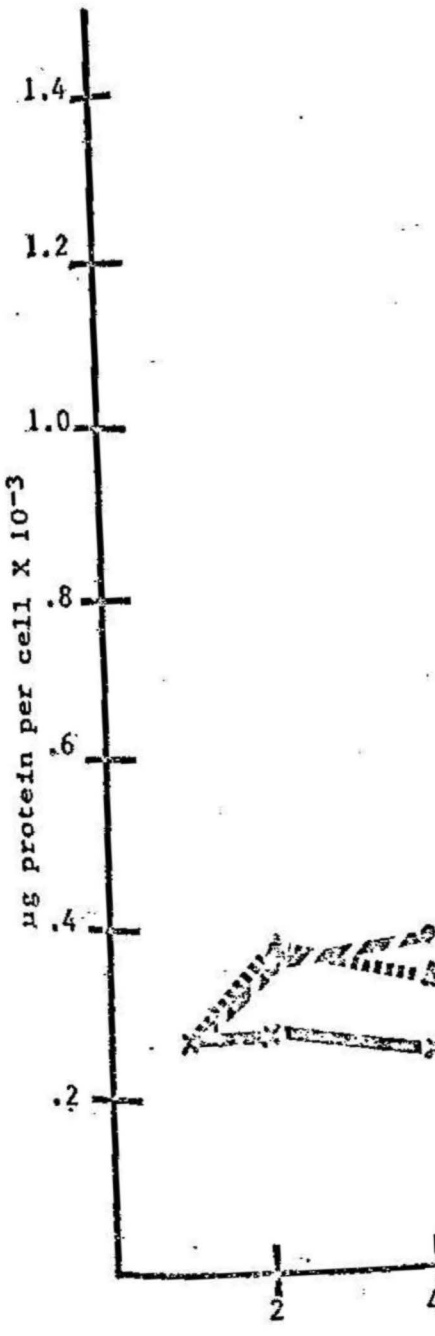
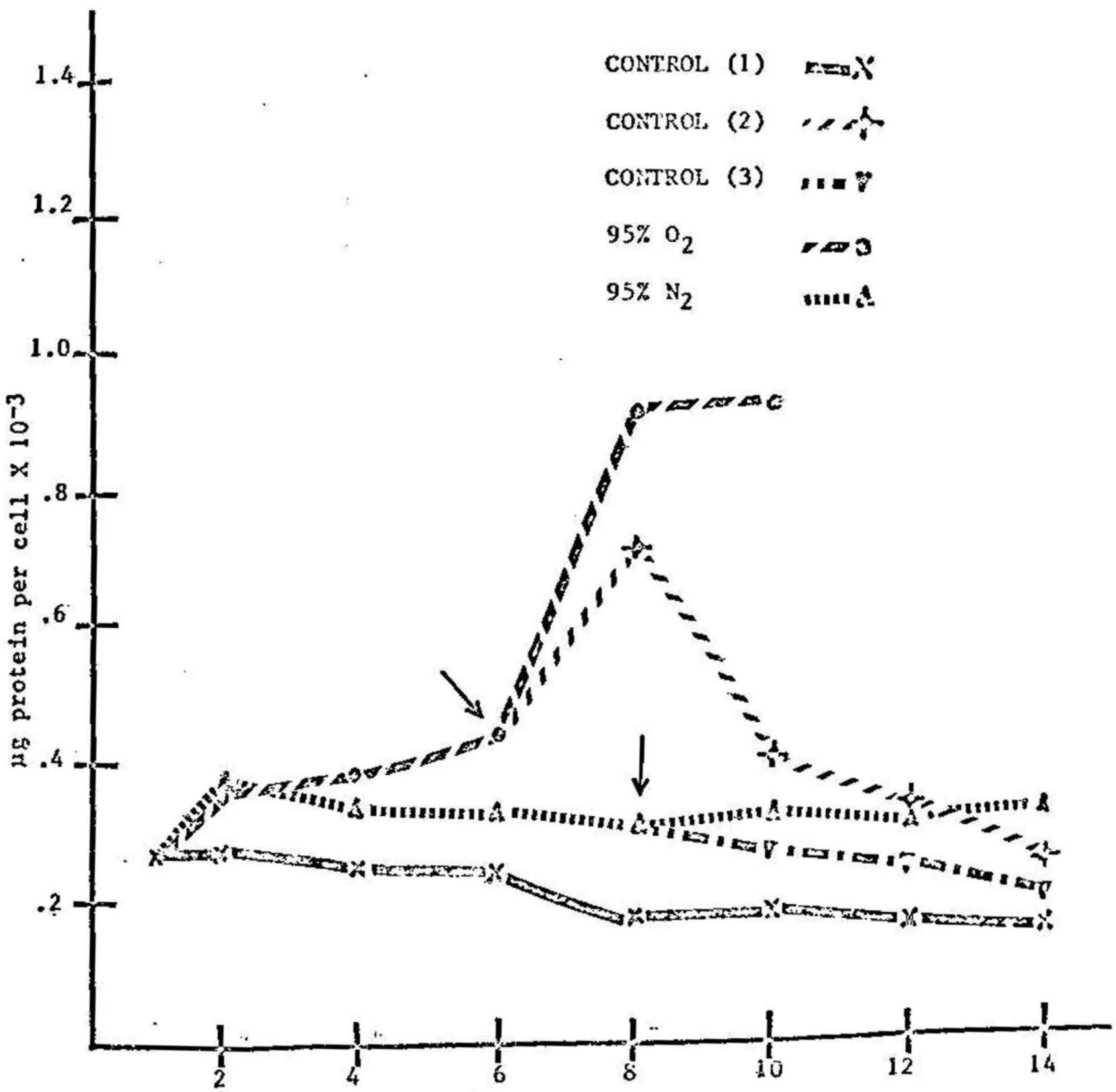


FIGURE 18. Compilation of 3 experiments showing amount of protein per cell in Low Line cells exposed to control conditions (95% air plus 5% CO_2), 95% O_2 plus 5% CO_2, or 95% N_2 plus 5% CO_2 and the effects of eventual exposure of the "damaged" cells under the latter two conditions to control conditions. Arrows indicate the time of this changeover.

FIGURE 18


CONTROL (1)
CONTROL (2)
CONTROL (3)
95% O_2
95% N_2
1.4
1.2
1.0
.8
.6
.4
.2
µg protein per cell X 10-3
2
4
6
8
10
12
14
TIME IN DAYS

control cultures (control-1) exhibit a rather steady although slight decrease in protein content throughout the experiment. In those cultured in 95% O_2 the amount of protein per cell shows a dramatic increase especially after day 6. In those cultures first exposed to 95% O_2 and then transferred to 95% air (control-2) on day 6, there is a continued increase in protein per cell. This occurs during the 48 hours following the changeover but subsequently it decreases sharply and approaches the amount found in the original control cells at the termination of the experiment. When subjected to 95% N_2, the cells exhibit a slight rise in protein content per cell the first day of treatment but thereafter it becomes constant and remains so throughout the experiment. Those in cultures first subjected to 95% N_2 which on day 8 was replaced with 95% air (control-3) demonstrate a steady decrease in protein content per cell and reaches a level closely resembling that found in the original controls (control-1) at the termination of the experiment.

Succinic Dehydrogenase Activity

In the histochemical demonstrations of the enzyme succinic dehydrogenase, the results obtained under the conditions of the experiment results in an intracellular deposit of a reddish-purple water-insoluble pigment. Low Line cells exposed to 95% air plus 5% CO_2 contain considerable quantities of succinic dehydrogenase (Fig. 19A). Naturally, this is most vivid when the cells become so numerous that they begin to "pile up" so that the stain appears to be much more intense.

In cells maintained in 95% N_2 for 6 days (Fig. 19B) this enzyme activity is approximately the same as that found in the controls. Also, these cells are much fewer in number than those in control cultures so that the additive effect of the stain is not as evident.

In contrast to this are the results obtained when cells are exposed to 95% O_2 for 6 days (Fig. 19C). Very little, if any stain is evident indicating that the succinic dehydrogenase activity is extremely low or lost completly. If following exposure for 6 days to 95% O_2, these cells are returned to 95% air (Fig. 19D) there is a reappearance of succinic dehydrogenase activity within 2 days after this changecver.

FIGURE 19

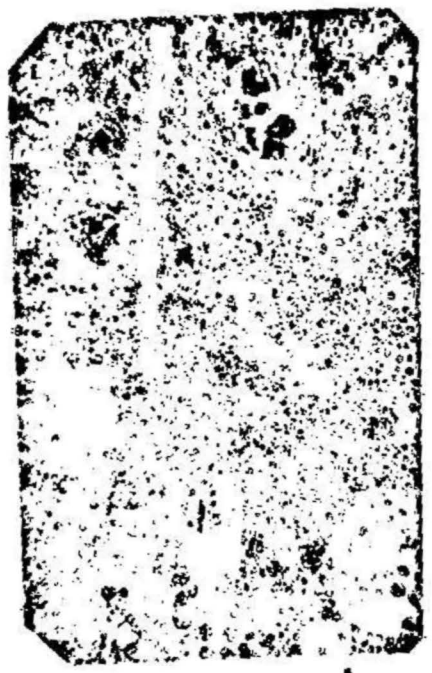
Low Line cells treated with Ditetrazolium chloride reagent, resulting in demonstration of succinic dehydrogenase activity.

A. Cells cultivated in an atmosphere of 95% air plus 5% CO_2 (control). (100X)

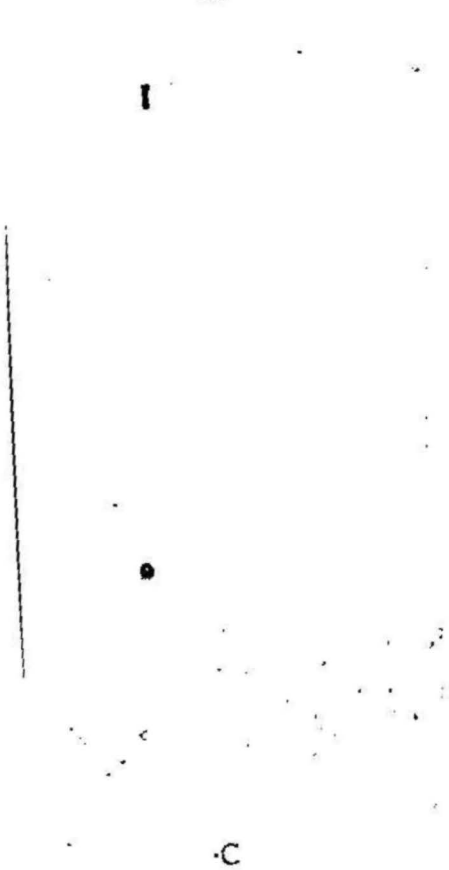
B. Cells subjected to an atmosphere of 95% N_2 plus 5% CO_2 for 6 days. (100X)

C. Cells exposed to an atmosphere of 95% O_2 plus 5% CO_2 for 6 days. (100X)

D. Cells 2 days after return to control conditions (95% air) following a 6 day exposure to 95% O_2. (100X)

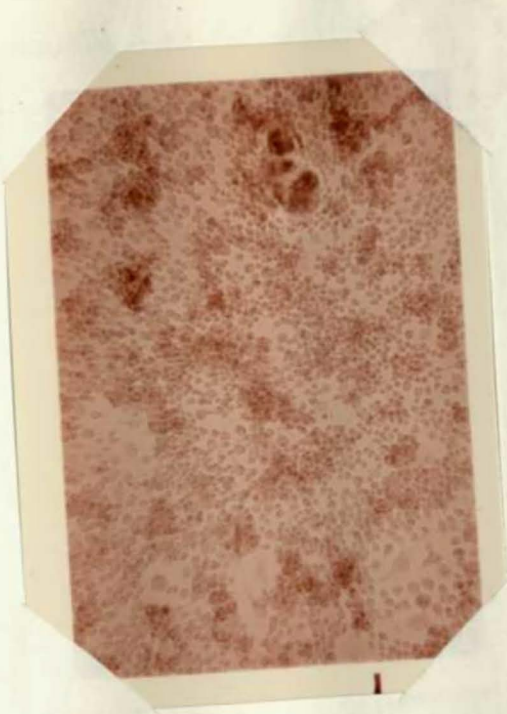


A



C

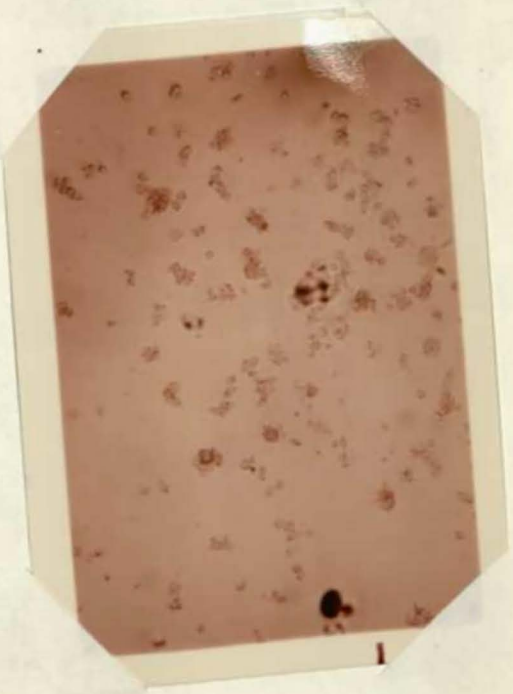
FIGURE 19



A

B

C



D

Cytochrome Oxidase Activity

The presence of cytochrome oxidase activity may be demonstrated histochemically by the technique of Burstone (1961) which results in the formation of a blue-black stain at the site of enzyme activity. This demonstration of cytochrome oxidase activity is clearly evident in Low Line cells exposed to an atmosphere of 95% air (Fig. 20A). Cells in 95% O_2 for 4 days (Fig. 20B) also show a definite positive response to this procedure although the cytotoxic effect of the high O_2 is quite evident when the morphologies of the cells in air and in 95% O_2 are compared.

Figure 21A is another photomicrograph indicating cytochrome oxidase activity in control (95% air) cells and Figure 21B is a photomicrograph taken of cells that had been exposed to 95% N_2 for 8 days. There is no demonstratable difference in their cytochrome oxidase content. The adversity of the low O_2 environment retards their proliferation so that the cells are less concentrated than those in the control cultures. As a result, it may appear that the enzyme is more concentrated in control cells which may be several cell layers in thickness. However, if the cells are examined individually, there appears to be very little, if any, difference.

Cells that were first exposed to 95% O_2 for 6 days and then returned to 95% air (Fig. 21C) continue to exhibit cytochrome oxidase activity and begin to revert to a "normal" morphology as seen in the original control cultures.

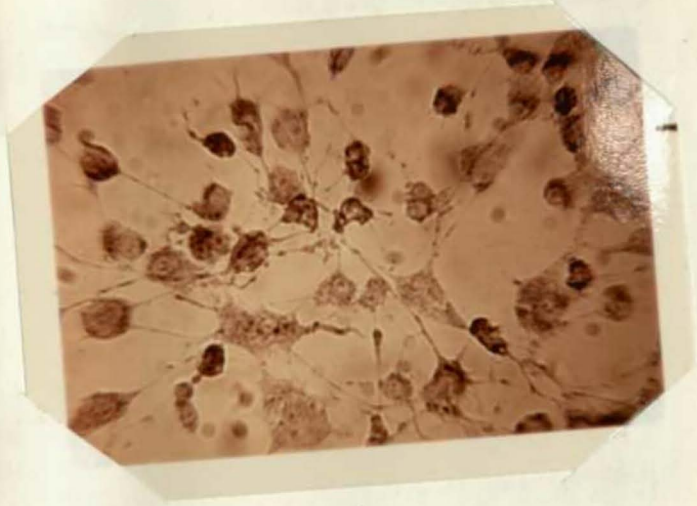
FIGURE 20

Histochemical demonstration of cytochrome oxidase in Low Line cells.

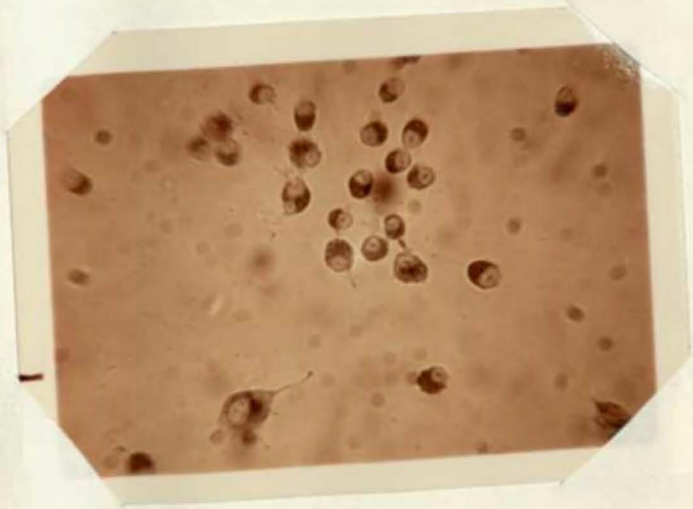
A. Cells exposed to 95% air plus 5% CO_2 (control). (430X)

B. Cells subjected to an atmosphere of 95% O_2 plus 5% CO_2 for 4 days. (430X)

FIGURE 20



A



B

FIGURE 21

Histochemical demonstration of cytochrome oxidase in Low Line cells.

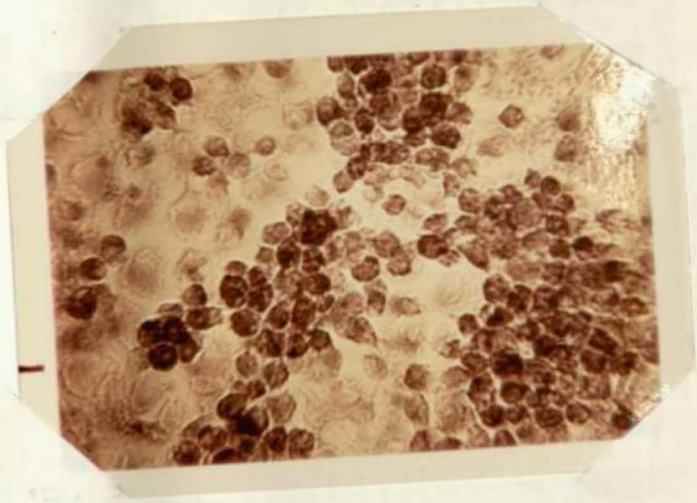
A. Cells exposed to 95% air plus 5% CO_2 (control) (430X)

B. Cells cultivated in an atmosphere of 95% N_2 plus 5% CO_2 for 8 days. (430X)

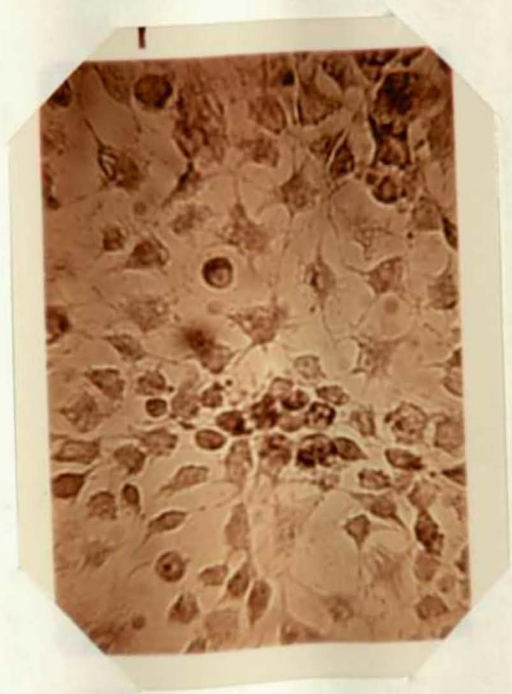
C. Cells 2 days after return to control conditions (95% air) following a 6 day exposure to 95% O_2. (430X)

B

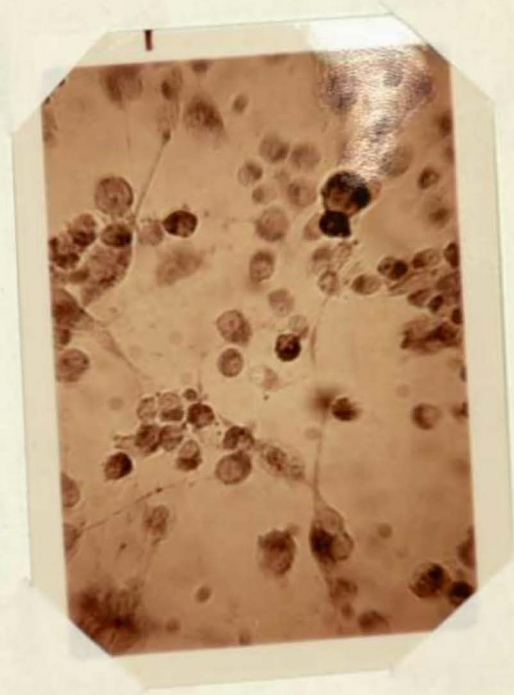
FIGURE 21



A



B



C

Cellular Lipid Accumulation

The Oil-Red-O staining technique was applied to Low Line cells for demonstrating lipid accumulation. Lipids, if present, will stain pink to bright red. In cells exposed to 95% air (control) the amount of lipid material is minimal (Fig. 22A).

On the other hand, cells cultivated in an atmosphere containing 95% O_2 (Fig. 22B) exhibit a fairly high degree of lipid accumulation. In those previously exposed to 95% O_2 and then returned to 95% air (Fig. 22C) a substantial decrease in the number of lipid droplets is observed. The cells also revert to a more "normal" morphology.

Exposure of Low Line cells to an atmosphere of 95% N_2 (Fig. 23A) results in an increase in lipid accumulation as is clearly seen in the photomicrograph. Also, the rather large size and bizzare morphology commonly observed in cells treated with low O_2 is evident. When the 95% N_2 is replaced with 95% air (Fig. 23B) the lipid droplets are diminished in a few days and the cells take on an appearance closely resembling that of the original controls (Fig. 22A).

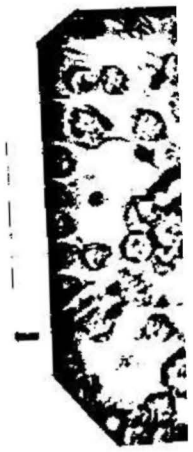
FIGURE 22

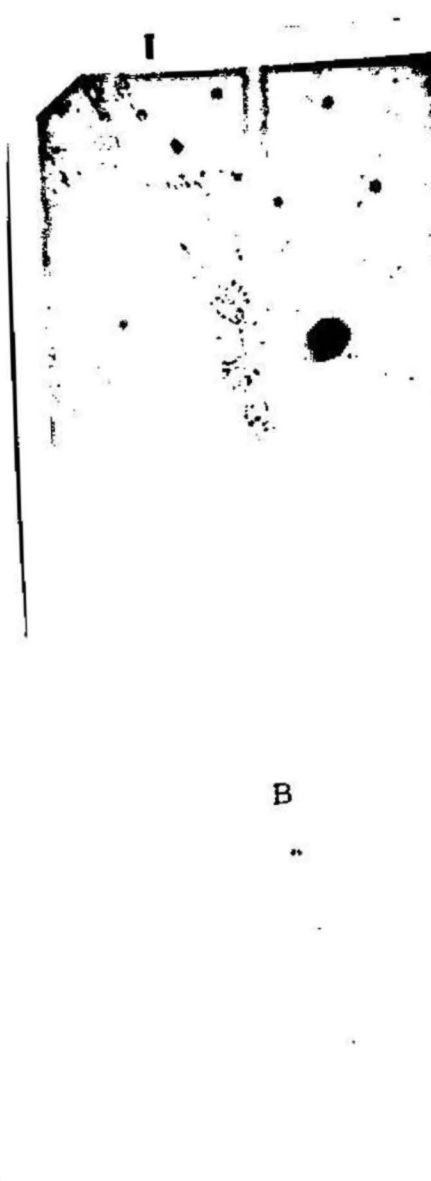
Lipid content of Low Line cells exposed to different environmental conditions.

A. Cells exposed to an atmosphere of 95% air plus 5% CO_2 (control). (430X)

B. Cells cultivated in an atmosphere of 95% O_2 plus 5% CO_2 for 4 days. (430X)

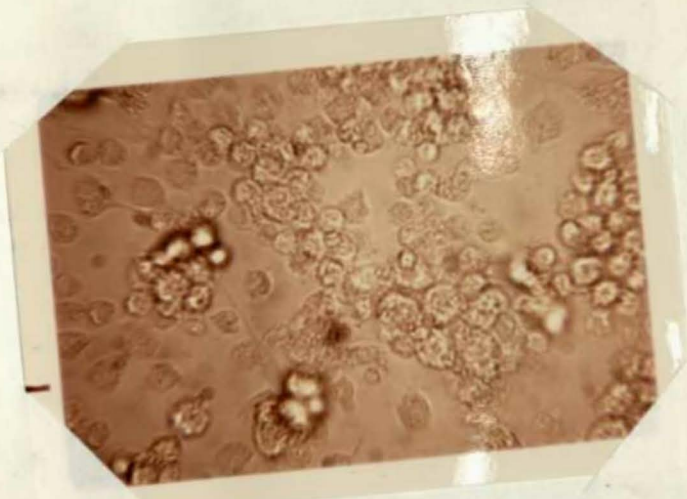
C. Cells first exposed to 95% O_2 for 6 days then to control conditions (95% air) for 4 days. (430X)





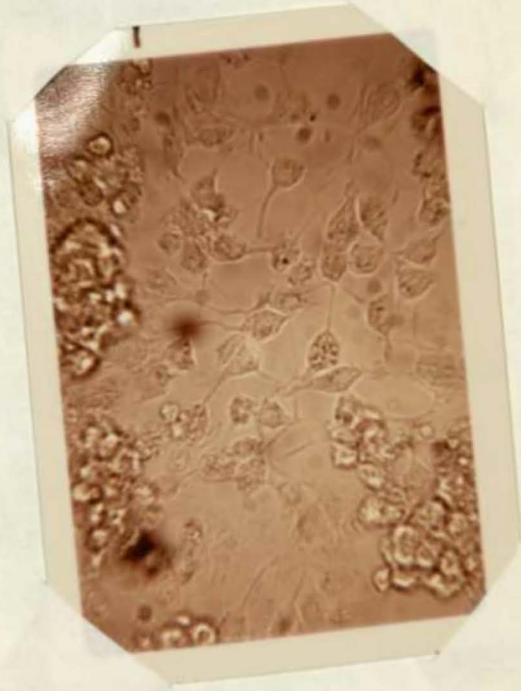
B

FIGURE 22



A

B



C

FIGURE 23

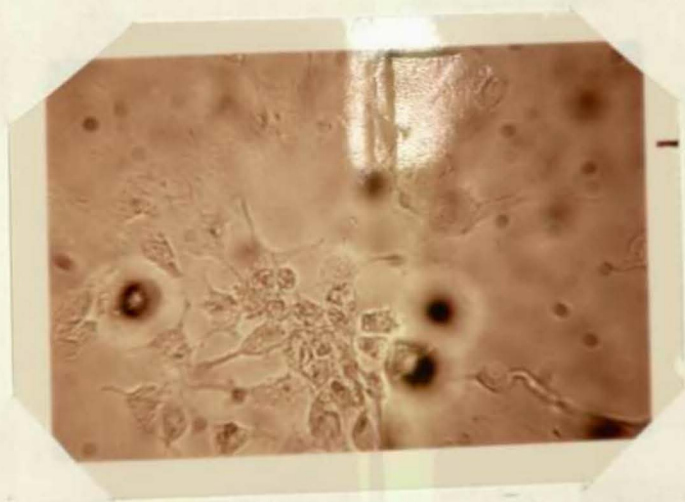
Lipid content of Low Line cells exposed to different environmental conditions.

A. Cells exposed to an atmosphere of 95% N_2 plus 5% CO_2 for 8 days. (430X)

B. Cells first exposed to 95% N_2 for 8 days then to control conditions (95% air) for 2 days. (430X)

FIGURE 23

A



B

DISCUSSION

DISCUSSION

The results of these experiments with Low Line cells verify the earlier investigations regarding the toxicity of high O_2 tensions and its effects on metabolic processes in tissue cells (Bonting and Jones, 1956; Rueckert and Mueller, 1960; Brosemer and Rutter, 1961; Pace et al., 1962).

Although different cell types vary significantly in their resistance to a continuous high O_2 environment, all the cell lines that have been investigated show a decrease in growth rate with increase in time of exposure. Similar results were obtained in the present studies with Low Line cells; also it was found that they will survive somewhere between 8 and 10 days. This survival time compares with that obtained by Pace et al., (1962) who reported that Strain L cells will live for as long as 14 days in 100% O_2. They also found that mouse liver cells will last for 9 days; human skin, 8 days; and HeLa cells, 3 days. In 95% O_2 plus 5% CO_2, only Strain L showed significant proliferation. Interestingly enough, Cooper,Burt, and Wilson (1958) found that O_2 levels as low as 25-30% are cytocidal to rabbit kidney cells grown in suspension.

Low Line cells, as is true for other cell lines, shift from a relatively high respiratory to a glycolytic metabolism when exposed to high or low O_2 tensions. In other words, the rate of glucose utilization, pyruvic and lactic

acid production increases considerably. Bonting and Jones (1956) found that carbohydrate metabolism of 15-day chick embryonic lung and intestinal cells cultured in 100% 0_2 is shifted partly to the glycolytic side, and that these cells become inactive after 4-6 days with a loss in total protein. They feel that this is due to an inactivation by the 0_2 of one or more of the respiratory enzymes followed by a gradual inactivation of one or more of the glycolytic enzymes.

Extensive experiments by Thomas et al., (1963) demonstrated that inhibition of carbohydrate metabolism by oxygen can occur rapidly in brain preparations in vitro. They observed that during a 30 minute incubation at 5 atm. 0_2 the production of radioactive $C0_2$ from C^{14}-glucose in rat brain homogenates is depressed to 20-30% of control values at 1 atm. They found that the production of labeled pyruvate from the labeled glucose is not apparently altered by the high 0_2 treatment, although the amount of $C^{14}0_2$ produced from the labeled pyruvate is markedly depressed. They conclude therefore the toxic effect of 0_2 is caused mainly by an inhibition of the enzyme or enzymes involved in pyruvate oxidation. This would explain the tremendous increases in pyruvate and lactate production in Low Line cells exposed to high 0_2 tensions as noted in the experiments presented here.

The idea that excessive elevation in O_2 tension destroys or inactivates certain enzymatic pathways is not new. The ability to detect changes in such pathways may be enhanced by studies employing hyperbaric O_2. Stadie and Haugaard (1945) found that the enzyme, succinic dehydrogenase, is inactivated in rat liver, kidney, and brain homogenates when subjected to 7 atmospheres of O_2. This inactivation proceeds rather slowly but is much slower when tissue slices are used rather than homogenates. Dickens (1946) also noted the sensitivity of succinic dehydrogenase to high O_2 tensions.

Succinic dehydrogenase is a flavoprotein with free sulfhydryl or SH groups. Enzymes which are especially sensitive to the inhibitory action of high O_2 are the so-called SH enzymes. On treatment with high O_2 the inactivation of these enzymes involves the formation of a disulfide form of the enzyme according to the equation:

$$2\ EnSH \underset{\text{Reduction}}{\overset{\text{Oxidation}}{\rightleftharpoons}} EnS\text{-}SEn + 2\ (H)$$

Active enzyme — Inactive enzyme

The inactive form can be reactivated by the addition of cysteine, or reduced glutathione. High concentrations of malonic and succinic acid also offer protection against

inactivation. This suggests that protection of sulfhydryl groups in enzymes by their substrates may play a role in the defense of the cell against the influence of oxygen and other oxidizing agents (Haugaard, 1968). Because of the inactivation of certain constituents of the citric acid cycle, cells cultivated in a high O_2 environment increase their glycolytic activity.

Lactate dehydrogenase (LDH) has been shown to be regulated by O_2 tension. This enzyme reversibly catalyzes the conversion of pyruvic acid to lactic acid. LDH in higher animals is composed of two kind of sub-units, which have been designated heart (H) and muscle (M) type (Cahn et al., 1962). These sub-units combine randomly to form enzymatically active tetramers.

Lindy and Rajasalmi (1966) found that the synthesis of the M sub-unit of LDH in chick embryos is suppressed by elevated O_2 tensions (40%) and promoted by lowered O_2 tension (15%). This tendency has also been observed by Johansson (1966,1967) using Chang Liver cells. He also reported that the total specific LDH activity is increased when the cells are exposed to an atmosphere of 95% N_2 plus 5% CO_2. Likewise, Adebonojo et al., (1961) demonstrated elevated LDH activity in Strain L and Ehrlich Ascites cells cultured under periodic anaerobiosis (16 hours per day) for up to 3 months. There findings are comparable to the LDH

results presented in this report on Low Line cells exposed to low O_2 tension.

Johannson (1967) observed no significant change in the activity of LDH within 24 hours when the cells were exposed to 95% O_2 plus 5% CO_2. This would seem to be in contrast with the results of the present study which indicate a highly elevated LDH activity in high O_2 concentration. However, Johansson's investigation was terminated within 24 hours while the work presented here was carried out over a much longer period of time (12 days). It is possible that had he carried his experiment over a longer period of time the toxicity of the high O_2 environment would have become more apparent and the results presented here may not have disagreed with those of his study.

High or low O_2 tensions also have an effect on nucleic acid synthesis of cells in vitro. The results presented here show a marked decrease in both the DNA and RNA content of the cells per ml. of culture medium under both high (95%) and low O_2, although this inhibition is much greater in the high O_2 condition. This compares favorably with the results of Matsuoka and Seno (1960) using Strain L and HeLa cells and Anderson et al., (1968) using human lymphocytes in vitro; both noted a decreased rate of DNA and RNA synthesis in cells per ml. of culture medium in hypoxic and hyperoxic environments with the effect much more marked in the latter

condition.

Drew et al., (1964) found that in 95% O_2 plus 5% CO_2 the incorporation of labeled thymidine into HeLa S3 cells is only half that of those in the control cultures at 24 hours. At the end of 48 hours no labeled thymidine is present inside the cells at all. Likewise, Answini and Nardone (1965) have shown that nucleic acid synthesis is definitely inhibited in Strain L cells after 24 hours exposure to high O_2 tension. This inhibition is much greater at 48 hours.

Rueckert and Mueller (1960) demonstrated a generalized reduction in the rate of cell division and in the biosynthesis of DNA , RNA and protein in HeLa cells exposed to 95% O_2. Thus, such an effect on nucleic acid synthesis might explain the fore-mentioned differences in growth and carbohydrate metabolism found in the present experiments on Low Line cells. When the amount of DNA and RNA is determined on a per cell basis this same general pattern is noted with one exception. The amount of RNA per cell in those exposed to 95% N_2 is somewhat higher than control cells. This is consistent with the observation that the cells grown under anaerobic conditions are generally much larger than controls and often show bizarre morphological shapes. Thus, mitosis may be inhibited while RNA replication and protein synthesis may not be as adversely effected.

As the recovery process advances in cells previously exposed to high or low O_2 the DNA and RNA per cell increases and actually exceeds the amount found in control cells. Later the amount of DNA and RNA per cell gradually approaches that of the original controls.

The amount of protein per ml. of culture medium in high or low O_2 environments compares with that reported for HeLa cells under like conditions (Rueckert and Mueller, 1960). The results, when plotted, follow the same pattern as that of the growth curves.

Low Line cells can survive and proliferate, at least for several weeks, in the virtual absence of O_2. This fact in itself does not necessarily indicate that the cells differ from mammalian cells growing in vivo (Sommer, 1964). Simply because mammalian systems may be obligatory aerobic it does not necessarily follow that individual cell types must require O_2 for growth or other function. It is known, for example, that the mammalian red cell does not contain cytochromes and consumes little if any O_2 under ordinary conditions (Brosemer and Rutter, 1961). However, Low Line cells normally consume O_2 when it is available (Woods et al., 1955). Possibly, then, the Low Line cells may be considered faculative anaerobes and compensate, at least partially, for the loss of respiratory energy in anaerobic conditions by accelerating the process of glycolysis. The complete

oxidation of glucose via the pentose shunt and citric acid cycle yields a total of 38 high energy bonds in the form of ATP, while only two ATP molecules are produced per molecule of glucose during anaerobic glycolysis. From this it is obvious that an increase consumption of glucose under anaerobic conditions would tend to counteract the lower yield of energy per molecule.

Woods et al., (1959) reported that glycolysis in Low Line cells increases in the absence of O_2 to the point of yielding about one-fourth the energy normally obtained by the aerobic catabolism of a lesser quantity of glucose; while a sister strain to the Low Line cells (termed High Line) can increase glycolysis under anaerobic conditions to the point of yielding about 85% of the energy normally obtained by the aerobic catabolism of a lesser quantity of glucose.

Clark (1964) contends that it is impossible to provide absolute anaerobic conditions using silicone-stoppered flasks. She calculated that enough O_2 reaches the cells through the stopper to supply the minimum requirements but she was successful in developing a technique by means of which Strain L cells could be subjected to strict anaerobiosis. From observations made under these conditions, she concluded that cells would not survive in 0% O_2. Other investigators have reported cellular survival in essentially 0% O_2 (Bonting and

Jones, 1956; Pace et al., 1962; Sommer 1964). Interesting as it may be, the importance of O_2 studies of this nature appears to rest in the relative influence of each tension, whether reduced or elevated, rather than the exactness of the tension. These influences can and have been observed. From the results presented here it can be seen that in many instances lactic acid production exceeds glucose utilization. This is especially evident in 0% O_2 (95% N_2 plus 5% CO_2) cultures. Brosemer and Rutter (1961) also found an excessive quantity of lactic acid production in cells exposed to 0% O_2. From where, then, does this excess lactic acid come? Obviously, it must come from other metabolic pathways than from those concerned with glucose metabolism.

Wilson et al., (1942), using chick embryo tissues, and Bailey et al., (1958), working with mouse lymphoblasts, found that lactic acid is formed in the presence or absence of glucose. Apparently, then, glucose is not essential for the production of lactic acid. Bailey et al., also observed that although glucose utilization decreases with increased incubation time, the amount of glycerol utilization increases with increased incubation time. This, then, could possibly account for at least part of the lactic acid production without glucose utilization.

Since lactic acid is the end product of glycolysis, quantitative analyses of this substance have been made to

determine glycolytic rate in cells. One fallacy in this procedure, however, is the fact that lactic acid may be utilized by the cells, therefore giving a false impression as to the actual rate of glycolysis. Wilson et al., (1942) suggested that lactic acid may not only be utilized, but it may especially favor growth in the absence of glucose. Bailey et al., (1958) found that cells survive in the absence of glucose for 48 hours but apparently utilize lactic acid present in the medium. Also, Munyon and Merchant (1959) found that lactic acid is taken up by the cells when glucose is present in either high or low concentrations.

Since the lactic acid produced by the cells may be utilized and since it may also be derived from sources other than glucose, it seems highly probable that one or more sources may be drawn upon to account for the fact that glucose utilization does not necessarily coincide with lactic acid production as shown in the results of these experiments.

As indicated earlier, there appear to be conflicting reports on the amount of glucose utilized according to the concentration in the medium. Wilson et al., (1942) reported that glucose is utilized in greatest quantities per unit time if the medium containes 100 mg.% than if it is in lower or high concentrations. Bonting and Jones (1956)

found that an increase in the concentration of glucose from 100 to 500 mg.% resulted in an increase in glucose utilization and lactic acid production. Cooper et al., (1958) found a glucose concentration of 600-800 mg.% to be most satisfactory. However, Gelfant (1959) observed that the greatest number of mouse epidermal mitoses occurred with a glucose concentration of .002 M (38 mg.%) and at an O_2 tension of 100%. Perhaps the conflicting reports appearing in the literature with references to the effects of O_2 on growth and metabolism could be due to differences in initial glucose concentrations.

Another finding in the current study is the massive accumulation of neutral fats in the form of cytoplasmic lipid globules under both high and low O_2 tensions. Noguchi et al., (1967) reported accumulation of large globules stainable with oil-red-O in the cytoplasm of several primary human cell lines exposed to hyperbaric oxygen for 24-72 hours. Earlier, Siegel et al., (1963) reported lipid droplets in HeLa cells subjected to elevated O_2 tensions.

This reaction may be a consequence of mitochondrial damage. Numerous studies on a variety of living systems have shown that enzymes associated with the mitochondria are involved in the toxic reaction to oxygen at high concentrations. Thomas and Neptune (1963) found that oxidation of glucose to carbon dioxide by rat brain homogenates and

isolated mitochondria was decreased in an environment of O_2 at 5 atmospheres compared to O_2 at 1 atmosphere. They observed that lactic and pyruvic acids accumulated and that the oxidations of pyruvate and a-ketoglutarate were inhibited at the high O_2 concentrations. Matsuoka and Seno (1960) using Strain L and HeLa cells reported swelling of mitochondria under high O_2 tension and pycnotic mitochondria under extremely low O_2 tension (0%).

As mentioned earlier, investigators have pinpointed the oxidation of pyruvic acid as one of the metabolic reactions inhibited by high O_2 tensions. If, as postulated by Dilworth (1962), the actual site of inhibition of the pyruvic acid oxidation system is below those sites generating acetyl coenzyme A, the latter substance, prevented from entering the oxidative pathway, may form fatty acids which can be stored in the cells as neutral fats. Upon removal of the toxic condition (high or low O_2) this inhibition is removed and the stored fat is metabolized as indicated by the results presented here.

In spite of its wide spread occurrence, the function of alkaline phosphatase is poorly understood. The enzyme is believed to play a role in absorption, as implied by its localization in the cell membrane area (Melnykovych et al., 1967) and by its increase, during neonatal development, in proportion to the absorptive capacity of the intestinal

mucosa (Clark, 1957).

Although repeated determination were made for alkaline phosphatase in Low Line cells during these investigations, we were unable to detect any significant alkaline phosphatase activity using both histological and colorimetric methods. These results are in agreement with Cox and Macleod (1962) who tested different tissue culture cell lines extensively for alkaline phosphatase content. They were able to demonstrate that epithelial cell lines either have constitutive alkaline phosphatase activity or it is inducible by exposure to hydrocortisone. The mechanism of this effect is not known. They also showed that fibroblasts cell lines from both human and mouse origin do not contain alkaline phosphatase activity nor could it be induced. Thus, their findings coincide with the aforementioned results in the present study on Low Line cells (mouse fibroblasts).

Acid phosphatase is usually considered to be associated with the lysosomes of the cell cytoplasm. The elevation in acid phosphatase activity under hyperoxic and hypoxic environments obtained in our present study is similar to that reported by Cristofalo et al., (1967). They observed this increase in acid phosphatase activity in WI-38 cells (human lung) during "aging" of the cells. Possibly the mechanism involved in this increase is the

same in both cases. However, in either case, further experimentation is necessary to ascertain if the increase in acid phosphatase activity represents a real increase in enzyme concentration per cell or an increase in the fragility of the lysosomal membrane which, during homogenization, permits more rapid diffusion of the substrate to the enzyme or vice versa.

The net effect of an increase in acid phosphatase activity is to lower the intracellular concentration of metabolically important phosphate esters and gradually shift the equilibrium in the cells away from synthesis and towards catabolism, thus resulting in a general deterioration of the cells (Cristofalo et al., 1967).

The effects of high O_2 tension on cytochrome oxidase in cultured cells, as reported here, confirms the results obtained in tissue homogenates exposed to elevated O_2 by Stadie and Haugaard (1945). They noted no effect on cytochrome oxidase by O_2 at 7 atmospheres. Likewise, the present study shows no noticeable reduction in cytochrome oxidase under 95% O_2.

Adebonojo et al., (1961) reported a decrease in cytochrome oxidase in Strain L cells when exposed to 95% N_2 plus 5% CO_2 for 16 hours a day over a period of three months. In the results presented here no noticeable effect was detected in the cytochrome oxidase content of Low Line

cells under anoxic conditions over a 12 day period. However, had we carried our experiments over a longer period of time the results of the two separate studies may have coincided.

According to Brosemer and Rutter (1961) recovery from the inhibitory effects of high O_2 tensions on growth in mammalian cells may be achieved only by lowering the O_2 tension within 48 hours. Longer periods of exposure to high O_2 concentrations will result in irreversible changes, including cellular degeneration. Similarly, Answini and Nardone (1965) found that recovery from the toxic effects of high O_2 tension on Strain L cells could be accomplished only if the environment of the cells was changed to a lower tension within 24-48 hours. According to these investigators, therefore, recovery is not possible after 48 hours exposure.

The work presented here was limited to observations on a cell line which has not, heretofore, been used in investigations concerned with possible recovery following lengthy exposure to anaerobic conditions or to high O_2 tension. The results give a few clues as to the primary effects of excessive O_2 concentrations or O_2 lack. They show that even after relatively lengthy exposures to 95% N_2 or to 95% O_2, the cells apparently recover completely if they are returned to 95% air plus 5% CO_2 and kept in

such an environment. Indications are that whatever damage is done to result in this inhibition, or inactivation by these conditions is soon repaired after re-exposure to normal conditions. Therefore, these changes are not as irreversible as some contend (Brosemer and Rutter; Answini and Nardone, 1965), at least not in Low Line cells under the conditions of the reported procedures.

SUMMARY AND CONCLUSIONS

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1. The effects of highly aerobic (95% O_2) as well as anaerobic (95% N_2) conditions on the proliferation and metabolism of Low Line cells (NCTC #2049) were studied.
2. In control (95% air + 5% CO_2) cultures, glucose utilization, pyruvic and lactic acid production per cell decreases as cell numbers increase.
3. Cells exposed to 95% O_2 show a steady decrease in growth while exhibiting a marked increase in glucose utilization, pyruvic and lactic acid production.
4. Cells exposed to 95% N_2 attain a low growth maximum which then remains fairly constant for at least 20 days. Glucose utilization, pyruvic and lactic acid production increases over that of the control (95% air) but reaches a plateau which then remains fairly constant.
5. Elevation in acid phosphatase and lactic dehydrogenase activities was noted in cells subjected to either high or low O_2 tension, with the elevation greater in the former condition.
6. Alkaline phosphatase activity was not detected in Low Line cells.
7. DNA, RNA, and protein content of the cells per ml. of culture medium in which the cells had grown

follows approximately the same pattern as that of the results obtained for growth under high and low O_2 tensions.

8. DNA, RNA, and protein content expressed per cell also follows the same general pattern as noted in the growth of the cells under high or low O_2 tension with some exceptions. The RNA per cell in cultures under Low O_2 tension is higher than in control cells. The amount of protein per cell under either high or low O_2 tensions is somewhat higher than in control cells.

9. Cytochrome oxidase activity appears not to be affected by either high or low O_2 environments.

10. Succinic dehydrogenase is inhibited by high O_2 tension but does not seem to be affected by low O_2.

11. Both high or low O_2 tensions initiate cellular lipid accumulation.

12. Cells exposed to 95% O_2 for up to 6 days recover from the toxic effects of this environment after being transferred to normal conditions (95% air). Shortly after this changeover all parameters tested returned to levels found in the control cultures. Cells exposed to 95% O_2 also show some signs of recovery after being transferred to 95% N_2. Those cultures which are continued in 95% O_2 die out soon after the

6th day of exposure.

13. Cells exposed to 95% N_2 for up to 10 days recover soon after they are transferred to 95% air. Shortly after this changeover all parameters tested returned to levels found in the control cultures. Cultures exposed to 95% N_2 and transferred to 95% O_2 on the 10th day of exposure, are affected by the high O_2 concentration almost immediately. Those cultures which were continued in 95% N_2 after the 19th day of exposure remain in a rather steady state.

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